Role of myoplasmic phosphate in contractile function of skeletal muscle: studies on creatine kinase-deficient mice

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1. Increased myoplasmic inorganic phosphate (P_i) has been suggested to have an important role in skeletal muscle fatigue, especially in the early phase. In the present study we used intact fast-twitch muscle cells from mice completely deficient in creatine kinase ($CK^{-/-}$) to test this suggestion. These $CK^{-/-}$ muscle cells provide a good model since they display a higher P_i concentration in the unfatigued state and fatigue without significant increase of P_i.

2. Tetanic contractions (350 ms duration) were produced in intact single muscle fibres. The free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured with the fluorescent indicator indo-1. The force–$[Ca^{2+}]_i$ relationship was constructed from tetani at different frequencies.

3. Compared with wild-type fibres, $CK^{-/-}$ fibres displayed lower force in 100 Hz tetani and at saturating $[Ca^{2+}]_i$ (i.e. 100 Hz stimulation during caffeine exposure), higher tetanic $[Ca^{2+}]_i$ during the first 100 ms of tetanic stimulation, reduced myofibrillar Ca^{2+} sensitivity when measurements were performed 100–200 ms into tetani, and slowed force relaxation that was due to altered cross-bridge kinetics rather than delayed Ca^{2+} removal from the myoplasm.

4. In wild-type fibres, a series of 10 tetani resulted in reduced tetanic force, slowed force relaxation, and increased amplitude of $[Ca^{2+}]_i$ tails after tetani. None of these changes were observed in $CK^{-/-}$ fibres.

5. Complementary experiments on isolated fast-twitch extensor digitorum longus muscles showed a reduction of tetanic force and relaxation speed in $CK^{-/-}$ muscles similar to those observed in single fibres.

6. In conclusion, increased P_i concentration can explain changes observed in the early phase of skeletal muscle fatigue. Increased P_i appears to be involved in both fatigue-induced changes of cross-bridge function and SR Ca^{2+} handling.

Phosphocreatine (PCr) breaks down to creatine (Cr) and inorganic phosphate (P_i) during high-intensity activity in skeletal muscle. *In vitro* studies on skeletal muscle preparations have shown that P_i affects both the contractile machinery and sarcoplasmic reticulum (SR) Ca^{2+} handling. Therefore, increased P_i has been suggested to be a key factor in skeletal muscle fatigue (Fitts, 1994; Allen *et al.* 1995). However, this suggestion has not been experimentally confirmed in intact muscle due to the lack of models where an increase in P_i is obtained without other metabolic changes that might also affect muscle function. Recently, mice completely deficient in creatine kinase ($CK^{-/-}$ mice) have been bred (Steeghs *et al.* 1997). CK catalyses phosphate exchange between the high-energy phosphates ATP and PCr via the reaction: $PCr + ADP + H^+ \rightleftharpoons Cr + ATP$. Compared with wild-type littermates, fast-twitch muscles of $CK^{-/-}$ mice have a higher P_i concentration at rest and they fatigue without PCr breakdown and associated P_i accumulation (Steeghs *et al.* 1997; Dahlstedt *et al.* 2000). Thus, fast-twitch $CK^{-/-}$ muscle provides a model for studying the functional effects of increased P_i in intact muscle.

Fatigue induced by repeated short tetani in intact single muscle fibres has been shown to occur in three phases (Allen *et al.* 1995). First there is a rapid decline of force to about 85% of the basal, which is accompanied by increased tetanic $[Ca^{2+}]_i$ (phase 1). Then follows a period of more stable tetanic force, the duration of which depends on the tetanic interval and fatigue resistance of the fibre (phase 2). Finally, both tetanic force and $[Ca^{2+}]_i$ fall relatively fast until fatiguing stimulation is stopped (phase 3). Several changes in muscle function that develop during phase 1 may be ascribed to increased P_i: (i) reduced cross-bridge force production and myofibrillar Ca^{2+} sensitivity (e.g. Millar & Homsher, 1990), (ii) increased tetanic $[Ca^{2+}]_i$ (e.g. Fruen *et al.* 1994), (iii) slowed force relaxation due to altered cross-bridge kinetics (Mulligan *et*

al. 1999), and (iv) slowed SR Ca^{2+} uptake (e.g. Dawson *et al.* 1980).

In the present study we compare force production and intracellular Ca^{2+} handling under resting conditions in single fast-twitch muscle fibres of $CK^{-/-}$ and wild-type mice. We also compare changes occurring early during fatiguing stimulation (i.e. during phase 1) in the two groups. Finally, complementary experiments were performed on isolated fast-twitch extensor digitorum longus (EDL) muscles. The results support an important role of increased P_i in changes of muscle function observed in early fatigue (phase 1) in fast-twitch muscle.

METHODS

Animals

$CK^{-/-}$ mice and their wild-type littermates were generated as described previously (Steeghs *et al.* 1997). Animals were housed at room temperature with 12 h : 12 h light–dark cycle. Food and water were provided *ad libitum*. Adult female mice were used and these were killed by rapid neck disarticulation and thereafter muscles were isolated. All procedures were approved by the local ethics committee.

Solutions

Experiments were performed at room temperature (24 °C). Isolated fibres or muscles were superfused with a Tyrode solution of the following composition (mM): NaCl, 121; KCl, 5.0; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4, 0.4; $NaHCO_3$, 24.0; EDTA, 0.1; glucose, 5.5; 0.2 % fetal calf serum was added to the solution to improve survival of single fibres. The solution was bubbled with 5 % CO_2–95 % O_2 to give a pH of 7.4. To assess force production at full activation of the contractile machinery, caffeine (5 mM) was added to the solution for 1 min prior to contraction and then rapidly removed.

Single fibre dissection, mounting and stimulation

Intact, single fibres were dissected from the flexor digitorum brevis (FDB) muscle of the hindlimb (Lännergren & Westerblad, 1987). The isolated fibre was mounted between an Akers 801 force transducer (SensoNor, Horten, Norway) and an adjustable holder at the length giving maximum tetanic force. Twitch and tetanic stimulation was achieved by trains of supramaximum current pulses (duration 0.5 ms) delivered via platinum plate electrodes lying parallel to the fibres. The duration of tetani was 350 ms. Under resting conditions, tetani of different frequencies were given at 1 min intervals. The effect of repeated contractions was assessed by giving ten 70 Hz tetani at 2.5 s intervals. Force is expressed in kPa, i.e. kN (m^2 cross-sectional area)$^{-1}$.

$[Ca^{2+}]_i$ and force measurements

$[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator indo-1 (Molecular Probes Europe B.V., Leiden, The Netherlands); the pentapotassium salt of indo-1 was microinjected into fibres, to avoid problems with loading of organelles. The fluorescence of indo-1 was measured with a system consisting of a xenon lamp, a monochromator and two photomultiplier tubes (PTI, Photo Med GmbH, Wedel, Germany). The excitation light was set to 360 ± 5 nm and the light emitted at 405 ± 5 and 495 ± 5 nm was measured. The ratio of the light emitted at 405 nm to that at 495 nm was translated to $[Ca^{2+}]_i$ as described elsewhere (Andrade *et al.* 1998).

After injection of indo-1, fibres were allowed to rest for at least 60 min. Thereafter some tetanic contractions were produced at 1 min intervals to ensure that force and $[Ca^{2+}]_i$ were stable. During contractions, fluorescence and force signals were sampled on-line and stored for subsequent data analysis. The steady-state force–$[Ca^{2+}]_i$ relationship in unfatigued fibres was established by measuring $[Ca^{2+}]_i$ and force in tetani produced at 10–100 Hz. To get the force at saturating $[Ca^{2+}]_i$, a 100 Hz tetanus in the presence of 5 mM caffeine was produced. $[Ca^{2+}]_i$–force curves were constructed in each fibre by using non-linear regression to fit data points to the following Hill equation:

$$P = P_{max}[Ca^{2+}]_i^N/(Ca_{50}^N + [Ca^{2+}]_i^N),$$

where P is the relative force, P_{max} is the force at full Ca^{2+} activation, Ca_{50} is the $[Ca^{2+}]_i$ giving 50 % of P_{max}, and N is a Hill coefficient that relates to the steepness of the function. Force–$[Ca^{2+}]_i$ curves were constructed from measurements of the mean force and $[Ca^{2+}]_i$ over the last 100 ms of stimulation (i.e. 250–350 ms into tetani). Force–$[Ca^{2+}]_i$ curves were also constructed from measurements performed 100–200 ms into tetani at 10–50 Hz (i.e. during the steep part of the curve) using the same P_{max} as above (see Fig. 2).

The force relaxation speed was assessed both on real and calcium-derived force records by measuring the time from the end of tetanic stimulation until force had decreased to 70 % of the maximum. In each fibre the calcium-derived force was constructed by converting tetanic $[Ca^{2+}]_i$ into force by means of the force–$[Ca^{2+}]_i$ curve (Westerblad & Allen, 1993*a*). The time to relax to 20 % of the maximum force was also measured. The two sets of measurements gave qualitatively similar results both for real and calcium-derived force, and for simplicity we will only report the time to relax to 70 % of the maximum force.

Indo-1 is a relatively slow Ca^{2+} indicator that cannot accurately follow rapid changes of $[Ca^{2+}]_i$. Therefore, kinetic corrections were performed as described previously (Westerblad & Allen, 1996*a*). We used an indo-1 Ca^{2+} dissociation rate (K_{off}) of 52 s^{-1}, which was obtained in *Xenopus* frog muscle fibres studied under conditions similar to the present (Westerblad & Allen, 1996*a*). With an intracellular indo-1 Ca^{2+} dissociation constant (K_D) of 283 nM (Andrade *et al.* 1998), this gives an indo-1 Ca^{2+} association rate (K_{on}) of 184 μM^{-1} s^{-1}. Two differences were observed when $[Ca^{2+}]_i$ records from tetanic contractions with and without kinetic correction were compared (see Fig. 1): a $[Ca^{2+}]_i$ transient at the onset of contraction was revealed with correction; uncorrected $[Ca^{2+}]_i$ records displayed a small delay (3–6 ms) during the first part of relaxation. Therefore, kinetically corrected indo-1 signals were used when measurements were performed during the first 100 ms of tetanic contraction and during the first 100 ms of relaxation. Also, kinetic correction was used to obtain the peak $[Ca^{2+}]_i$ with a single stimulation pulse (twitch). Measurements of $[Ca^{2+}]_i$ at rest, between 100 and 350 ms of tetanic contraction, and during the slow decay of $[Ca^{2+}]_i$ after the end of tetani were not significantly affected by kinetic correction.

Whole muscle experiments

Intact EDL muscles were dissected from one leg in each animal. Small stainless-steel loops were tied to the tendons of the muscle with thin nylon thread. The muscle was then mounted between a force transducer and an adjustable hook, which allowed the muscle to be stretched to the length giving maximum tetanic force. Tetanic stimulation was produced by applying supramaximal current pulses (0.5 ms duration) via plate electrodes lying on each side of the muscle. After obtaining the length yielding maximal tetanic force, the muscle was allowed to rest for 30 min. The contractile function in the unfatigued state was established by producing a 100 Hz, 300 ms tetanus. The effect of repeated tetanic stimulation was studied by giving a series of 10 tetani at 2 s intervals. Tetanic force and relaxation time were measured as described above for single fibres. The cross-sectional area was not measured in EDL muscles and forces are expressed relative to muscle dry weight. Muscle length was not measured but no consistent differences between wild-type and $CK^{-/-}$ muscles were noted during mounting. Therefore, forces expressed

relative to muscle dry weight should provide a reasonable measure of force per cross-sectional area.

There are technical reasons for using different muscles in the single fibre and whole muscle experiments. Muscle fibres of the FDB muscle are much shorter than EDL fibres, which is advantageous during single fibre dissection. However, the FDB muscles are multipennate and have three distinctly different distal tendons, which together with the short fibre length make them less suitable for whole muscle experiments. Mouse FDB and EDL muscles contain almost exclusively fast-twitch, type 2 fibres (Raymackers *et al.* 2000) and twitch kinetics of the present single FDB fibres and EDL muscles are very similar (Dahlstedt *et al.* 2000). Thus results from single FDB fibres and whole EDL muscles would give a fair account of the function of fast-twitch muscle in $CK^{-/-}$ and wild-type mice.

Statistics

Values are presented as means ± S.E.M. Student's unpaired *t* tests were used to determine significant differences between $CK^{-/-}$ and wild-type muscle; paired *t* tests were used when analyses were performed within each group. The significance level was set at 0.05 throughout.

RESULTS

Tetanic $[Ca^{2+}]_i$ and force

Experiments were performed on seven $CK^{-/-}$ and six wild-type single muscle fibres. Figure 1 shows representative records of $[Ca^{2+}]_i$ and force obtained from 100 Hz tetani produced in a wild-type fibre (Fig. 1*A*) and in a $CK^{-/-}$ fibre (Fig. 1*B*). Tetanic force was markedly lower in the $CK^{-/-}$ fibre despite the fact that tetanic $[Ca^{2+}]_i$ was, if anything, higher in this fibre. Mean data from 100 Hz tetani showed a significantly lower force in $CK^{-/-}$ fibres (241 ± 14 kPa) than in wild-type fibres (329 ± 23 kPa). Measurements of resting $[Ca^{2+}]_i$, performed immediately before tetanic stimulation, did not show any significant difference between $CK^{-/-}$ fibres (72.6 ± 5.7 nM) and wild-type fibres (70.0 ± 5.8 nM). Mean tetanic $[Ca^{2+}]_i$, measured during the last 100 ms of stimulation, was not significantly different between $CK^{-/-}$ fibres (1.58 ± 0.16 μM) and wild-type fibres (1.75 ± 0.21 μM). However, the shape of the tetanic $[Ca^{2+}]_i$ records differed between the groups (see Fig. 1). Thus, $[Ca^{2+}]_i$ increased significantly during the tetanic plateau in wild-type fibres with a slope of 1.43 ± 0.53 μM s^{-1} (measured in each fibre as the difference between mean $[Ca^{2+}]_i$ during the last and the first 100 ms of stimulation), whereas it tended to fall in $CK^{-/-}$ fibres (slope −1.09 ± 0.65 μM s^{-1}). Hence the mean tetanic $[Ca^{2+}]_i$ during the first 100 ms of stimulation was higher in $CK^{-/-}$ fibres (1.85 ± 0.30 μM) than in wild-type fibres (1.40 ± 0.11 μM), but this difference was not significant ($P = 0.21$). A similar pattern was observed at all tetanic stimulation frequencies; for instance, analysis of $[Ca^{2+}]_i$ records from

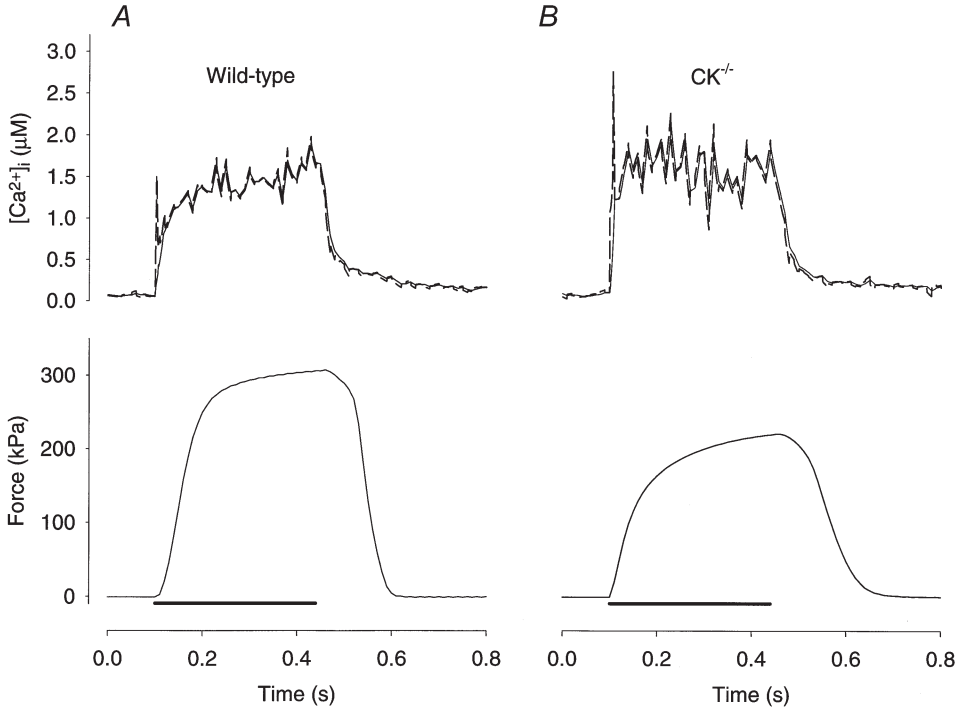

Figure 1. Tetanic force is markedly smaller in $CK^{-/-}$ fibres than in wild-type fibres despite similar or even higher $[Ca^{2+}]_i$

Typical records of $[Ca^{2+}]_i$ and force from 100 Hz tetani produced in a wild-type fibre (*A*) and a $CK^{-/-}$ fibre (*B*). $[Ca^{2+}]_i$ records are shown both with (dashed line) and without (continuous line) kinetic correction of the indo-1 signal. Observe that kinetic correction reveals a $[Ca^{2+}]_i$ transient at the onset of contraction, and that the decrease of $[Ca^{2+}]_i$ at the end of stimulation is slightly delayed without correction. Horizontal bars below force records show period of stimulation.

50 Hz tetani showed a significantly higher $[Ca^{2+}]_i$ during the first 100 ms of stimulation in $CK^{-/-}$ fibres (1.02 ± 0.09 μM) than in wild-type fibres (0.77 ± 0.05 μM), whereas there was no difference during the last 100 ms (0.94 ± 0.06 *vs.* 0.96 ± 0.07 μM). To further study the initial Ca^{2+} release, twitches were produced and the peak $[Ca^{2+}]_i$ measured. Peak $[Ca^{2+}]_i$ was significantly higher in $CK^{-/-}$ fibres (3.22 ± 0.41 μM) than in wild-type fibres (2.11 ± 0.20 μM).

The maximum force-generating capacity was assessed by producing 100 Hz tetani in the presence of 5 mM caffeine. Application of caffeine had similar effects on $CK^{-/-}$ and wild-type fibres and increased tetanic $[Ca^{2+}]_i$ by about 200% and force by about 7%. Thus, tetanic force at saturating $[Ca^{2+}]_i$ was significantly lower in $CK^{-/-}$ fibres (257 ± 15 kPa) than in wild-type fibres (353 ± 27 kPa).

Force–$[Ca^{2+}]_i$ relationship

The force–$[Ca^{2+}]_i$ relationship was determined by measuring force and $[Ca^{2+}]_i$ in tetanic contractions of different frequencies. Figure 2 shows representative $[Ca^{2+}]_i$ and force records from 40 Hz tetani, which lie on the steep part of the force–$[Ca^{2+}]_i$ relationship, from a wild-type fibre (Fig. 2*A*) and a $CK^{-/-}$ fibre (Fig. 2*B*). Again, the shape of tetanic $[Ca^{2+}]_i$ is different in the two fibres, with a gradual increase during the tetanus in the wild-type fibre and no obvious change in the $CK^{-/-}$ fibre. The shape of the force records, on the other hand, is more similar, with a gradual increase in both fibres, albeit the initial force increase is somewhat faster in the $CK^{-/-}$ fibre. This difference between the fibres affects the force–$[Ca^{2+}]_i$ relationship, which is illustrated in Fig. 2*C* and *D*. In the wild-type fibre, the force–$[Ca^{2+}]_i$ relationship did not depend on measurements being performed early during the tetanus (i.e. between 100 and 200 ms of stimulation; ○) or towards the end (i.e. between 250 and 350 ms of stimulation; ●). However, in the $CK^{-/-}$ fibre, the force–$[Ca^{2+}]_i$ relationship was shifted to the right with measurements early during the contraction as compared with measurements towards the end. The same pattern was observed in the other fibres and mean data of the force–$[Ca^{2+}]_i$ relationship (Table 1) show similar Ca_{50} values when measurements were performed late. With early measurements, however, there was a significantly higher Ca_{50} value in $CK^{-/-}$ fibres than in wild-type fibres. Table 1 also shows that P_{max} was significantly lower in $CK^{-/-}$ fibres than in wild-type fibres.

Tetanic relaxation speed

The tetanic force records in Fig. 1 show a slower relaxation in the $CK^{-/-}$ fibre. This was a consistent finding and mean data show about 30% longer relaxation time in $CK^{-/-}$ fibres as compared with wild-type fibres (Table 1). To investigate the mechanism behind this difference between the groups, calcium-derived force records were produced from $[Ca^{2+}]_i$ records and the force–$[Ca^{2+}]_i$ relationship. Representative force records from the relaxation phase of a wild-type fibre and a

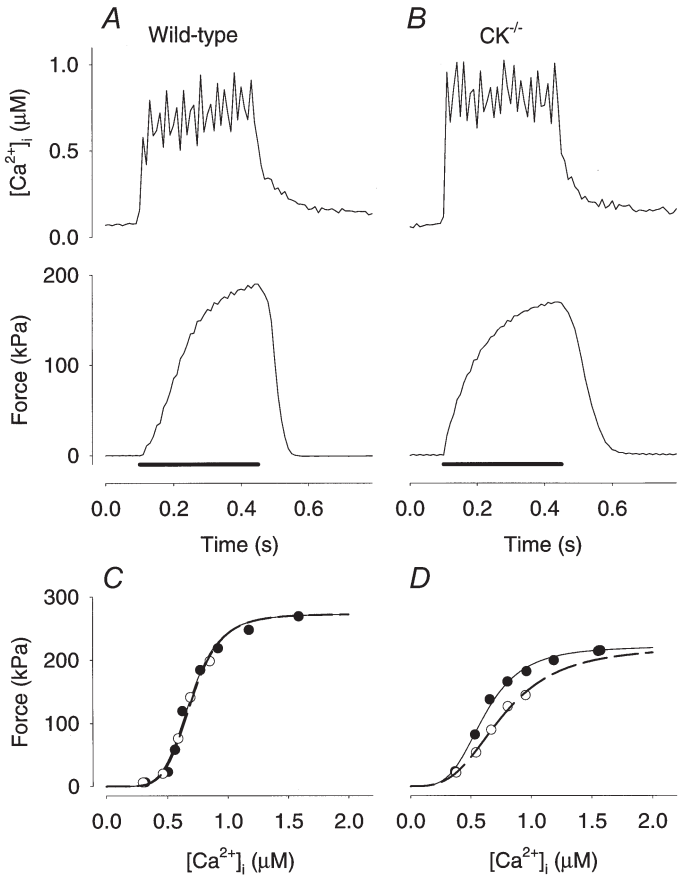

Figure 2. The myofibrillar Ca^{2+} sensitivity is lower in $CK^{-/-}$ fibres when measurements are performed early in the tetanus

Representative $[Ca^{2+}]_i$ and force records from 40 Hz tetani produced in a wild-type fibre (*A*) and a $CK^{-/-}$ fibre (*B*). Period of stimulation is indicated by bar below force records. Observe the different tetanic $[Ca^{2+}]_i$ patterns, with a gradual increase in the wild-type fibre but not in the $CK^{-/-}$ fibre. This has an impact on the force–$[Ca^{2+}]_i$ curves from the same fibres, which are shown in *C* (wild-type fibre) and *D* ($CK^{-/-}$ fibre). ○ (connected by dashed line) and ● (connected by continuous line) refer to measurements performed 100–200 ms and 250–350 ms into tetani, respectively. Note that while the two force–$[Ca^{2+}]_i$ curves were similar in the wild-type fibre, there was a clear shift to the right with early measurements in the $CK^{-/-}$ fibre.

$CK^{-/-}$ fibre are shown in Fig. 3. While relaxation of real force was slower in the $CK^{-/-}$ fibre, there was no marked difference in the relaxation speed of calcium-derived force. This was a general finding and the results are summarised in Table 1. The calcium-derived forces used in Table 1 were constructed using the force–$[Ca^{2+}]_i$ relationship obtained towards the end of tetani. Qualitatively similar results were achieved when the force–$[Ca^{2+}]_i$ relationship obtained earlier in the tetanus was used to construct calcium-derived force, although calcium-derived relaxation then became somewhat faster, especially in $CK^{-/-}$ fibres (data not shown).

Table 1. Force–$[Ca^{2+}]_i$ relationships and relaxation times in wild-type and CK−/− single fibres

	Wild-type ($n = 6$)	$CK^{-/-}$ ($n = 7$)
Force–$[Ca^{2+}]_i$ relationship		
250–350 ms into tetani		
P_{max} (kPa)	331 ± 21	249 ± 15*
Ca_{50} (μM)	0.65 ± 0.05	0.65 ± 0.03
N	5.74 ± 0.83	4.65 ± 0.49
100–200 ms into tetani		
Ca_{50} (μM)	0.69 ± 0.05	0.79 ± 0.02*
N	5.47 ± 0.68	3.85 ± 0.37
Relaxation time		
Real force (ms)	73.0 ± 5.2	96.3 ± 6.6*
Calcium-derived force (ms)	18.7 ± 1.7	20.0 ± 2.6

* Significant difference between wild-type and $CK^{-/-}$ fibres ($P < 0.05$). The relaxation time was measured in 100 Hz tetani as the time from the end of stimulation until force decreased to 70% of the maximum.

Changes in $[Ca^{2+}]_i$ and force due to 10 repeated tetani

A series of ten 70 Hz tetani was produced to study changes of $[Ca^{2+}]_i$ and force in early fatigue, i.e. during phase 1. In the tenth tetanus the force was reduced to 89 ± 2% of control in wild-type fibres, whereas it was not significantly changed in $CK^{-/-}$ fibres (104 ± 2%). However, absolute forces were still lower in $CK^{-/-}$ fibres (222 ± 10 kPa) than in wild-type fibres (271 ± 18 kPa). In the first tetanus, $[Ca^{2+}]_i$ (measured as the mean over the first 100 ms of stimulation) was significantly higher in $CK^{-/-}$ fibres (1.40 ± 0.18 μM) than in wild-type fibres (0.91 ± 0.06 μM). Tetanic $[Ca^{2+}]_i$ increased significantly during the series of tetani in wild-type fibres whereas it did not change in $CK^{-/-}$ fibres. Thus in the tenth tetanus, tetanic $[Ca^{2+}]_i$ was similar in $CK^{-/-}$ fibres (1.34 ± 0.14 μM) and wild-type fibres (1.24 ± 0.10 μM).

The relaxation speed was also measured at the start and at the end of the series of 10 tetani. In wild-type fibres there was a highly significant increase of the relaxation time (31 ± 4%) in the tenth tetanus. In $CK^{-/-}$ fibres, on the other hand, there was no significant difference between the relaxation times in the first and tenth tetanus. This means that in the tenth tetanus, relaxation times in wild-type and $CK^{-/-}$ fibres were not significantly different (85.8 ± 5.4 ms *vs.* 83.6 ± 7.0 ms; note that repeated 70 Hz tetani were used and at this stimulation frequency relaxation times of unfatigued fibres were slightly shorter than with 100 Hz tetani).

Tails of elevated $[Ca^{2+}]_i$ after the end of tetanic stimulation may be used to assess the function of the SR Ca^{2+} pumps (Klein *et al.* 1991). Ten repeated tetani resulted in increased tails of $[Ca^{2+}]_i$ in wild-type fibres (Fig. 4*A*). Measurements in these fibres, performed as the mean over 100 ms at 0.5, 1 and 2 s after the end of tetanic stimulation, showed significant increases of about 15 nM in the tenth as compared with the first tetanus. On the other hand, 10 repeated tetani had no significant effects on tails of $[Ca^{2+}]_i$ in $CK^{-/-}$ fibres (Fig. 4*B*). In the tenth tetanus, tails of $[Ca^{2+}]_i$ were similar in wild-type and $CK^{-/-}$ fibres; for instance, at 0.5 s after the end of tetanic stimulation $[Ca^{2+}]_i$ was 160 ± 7 nM in wild-type fibres and 162 ± 15 nM in $CK^{-/-}$ fibres.

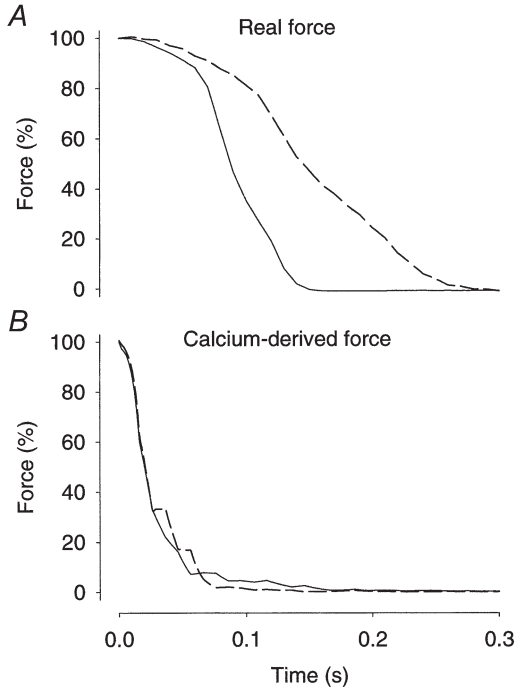

Figure 3. Force relaxation is markedly slower in $CK^{-/-}$ fibres than in wild-type fibres

A, real force records of the relaxation phase of 100 Hz tetani obtained in a wild-type fibre (continuous line) and a $CK^{-/-}$ fibre (dashed line). *B*, calcium-derived force records from the same fibres produced from tetanic $[Ca^{2+}]_i$ records and their respective force–$[Ca^{2+}]_i$ curves. The time axis starts at the end of tetanic stimulation. Note that the markedly slower relaxation in the $CK^{-/-}$ fibre is not accompanied by a slowed relaxation of calcium-derived force.

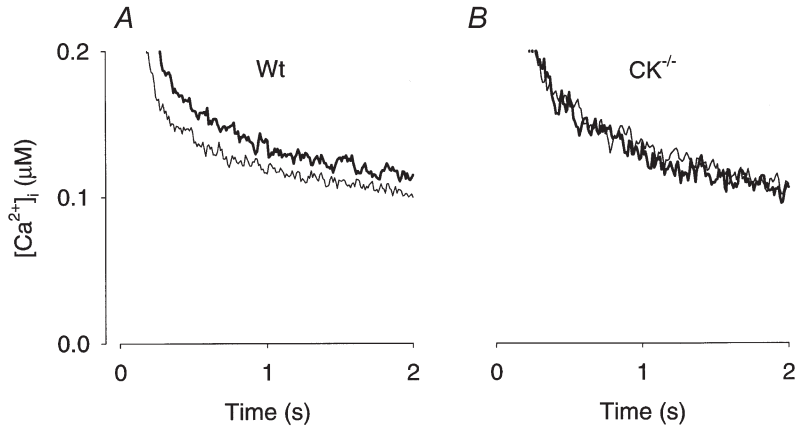

Figure 4. The amplitude of tails of elevated $[Ca^{2+}]_i$ is increased after 10 repeated tetani in wild-type but not in $CK^{-/-}$ fibres

Mean $[Ca^{2+}]_i$ records from the relaxation phase of wild-type fibres (Wt; *A*) and $CK^{-/-}$ fibres (*B*). Records were obtained in the first (thin line) and tenth (thick line) repeated tetanus.

Whole muscle experiments

Experiments were performed on five EDL muscles from $CK^{-/-}$ and wild-type mice, respectively. Representative force records from 100 Hz tetani produced in EDL muscles are shown in Fig. 5. Tetanic force was about 25% lower in EDL muscles from $CK^{-/-}$ mice (90 ± 6 N (g dry weight)$^{-1}$) as compared with the wild-type (118 ± 7 N (g dry weight)$^{-1}$). Furthermore, the relaxation time was significantly longer in $CK^{-/-}$ EDL muscles (56.0 ± 4.0 ms *vs.* 30.4 ± 3.1 ms in wild-type). Thus in the unfatigued state, the differences in contractile performance between $CK^{-/-}$ and wild-type EDL muscles are very similar to those observed in the single fibres.

We also measured force and relaxation speed after ten 70 Hz tetani in EDL muscles. Compared with the first fatiguing tetanus, force in the tenth tetanus was reduced to 84 ± 1% in wild-type and 92 ± 2% in $CK^{-/-}$ EDL muscles. In the tenth tetanus the relaxation time was increased by 67 ± 8% in wild-type muscles and by 45 ± 5% in $CK^{-/-}$ muscles. Thus, a series of 10 tetani caused a reduction of tetanic force and a slowing of relaxation in both groups, but the changes were significantly smaller in $CK^{-/-}$ muscles.

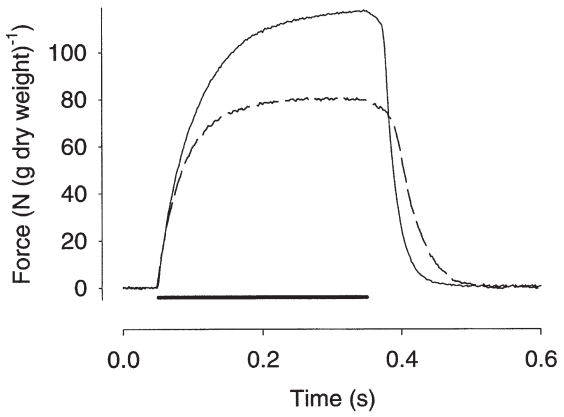

Figure 5. Tetanic force is smaller and relaxation slower in $CK^{-/-}$ EDL muscles

Typical force records from 100 Hz tetani produced in EDL muscles from wild-type (continuous line) and $CK^{-/-}$ (dashed line) mice. The period of stimulation is indicated below the records.

DISCUSSION

Increased P_i is considered to be an important cause of skeletal muscle fatigue. Specifically, changes in early fatigue, i.e. during phase 1, have been ascribed to increased P_i. However, lack of suitable models has prevented direct tests of this proposal in intact muscle. In the present study, we used muscles from $CK^{-/-}$ mice to investigate the effect of increased P_i on the function of unfatigued muscle and compared the results with those from early fatigue. $CK^{-/-}$ muscle provides a good experimental model since under resting conditions, there is a markedly higher P_i in fast-twitch EDL muscles of $CK^{-/-}$ animals than in wild-type (Dahlstedt *et al.* 2000). Furthermore, $CK^{-/-}$ muscles do not display any marked increase of P_i during fatigue (Steeghs *et al.* 1997; Dahlstedt *et al.* 2000) or during ischaemia (in't Zandt *et al.* 1999). It should be noted that rested $CK^{-/-}$ muscle also displays changes in metabolites other than P_i. For instance, ATP and PCr concentrations are lower and the Cr concentration is higher in $CK^{-/-}$ as compared with wild-type muscle (Steeghs *et al.* 1997; Dahlstedt *et al.* 2000). However, the differences in these metabolites are relatively modest and would have little effect on force production (Godt & Nosek, 1989). Furthermore, intracellular pH, which has a large effect on force production at the temperature used in the present experiments (Westerblad *et al.* 1997), is similar in rested $CK^{-/-}$ and wild-type muscles (Steeghs *et al.* 1997). Thus, $CK^{-/-}$ muscle provides a good model to study contractile effects of increased P_i, but some modulating role of other metabolic differences between $CK^{-/-}$ and wild-type muscles cannot be ignored.

Reduction of cross-bridge force production

When intact muscle fibres are fatigued by repeated tetanic stimulation, there is a reduction of tetanic force already after a few tetani, which has been ascribed to increased myoplasmic P_i (Westerblad & Allen, 1992). This

suggestion is strongly supported by the present results. Tetanic force was markedly lower in rested single fibres and EDL muscles of $CK^{-/-}$ mice, which are fast-twitch and have a higher P_i than wild-type fibres (Dahlstedt *et al.* 2000). Moreover, the decline in tetanic force during phase 1 is not present in $CK^{-/-}$ single fibres, which do not accumulate P_i. Additional support for a coupling between force production and P_i concentration in intact muscle comes from previous studies where a *reduced* P_i concentration is associated with *increased* force production (Phillips *et al.* 1993; Bruton *et al.* 1997). In this context it should be noted that studies of P_i effects on force production in mammalian muscle, including the present study, have generally been performed at temperatures well below body temperature. There are results showing that the force-depressing effect of P_i is smaller at high temperature (Dantzig *et al.* 1992), but at 30 °C increased P_i still gives a marked force depression in skinned, fast-twitch muscle fibres (Puchert *et al.* 1999).

Skinned fibre experiments demonstrate that increased P_i reduces cross-bridge force production (Pate & Cooke, 1989; Millar & Homsher, 1990; Potma & Stienen, 1996). Data from these skinned fibre experiments can be used to assess whether the reduction of force observed in fast-twitch $CK^{-/-}$ muscle is likely to be due to increased P_i. Translating values of P_i in rested EDL muscles of $CK^{-/-}$ (22.6 μmol (g dry weight)$^{-1}$) and wild-type (11.1 μmol (g dry weight)$^{-1}$) (Dahlstedt *et al.* 2000) into millimolar (mM), assuming two litres of intracellular water per kilogram of dry tissue (Sahlin *et al.* 1983), gives a P_i concentration of about 11.3 and 5.6 mM, respectively. Using data from skinned muscles, this increase in P_i should decrease force by 10–15 % (Pate & Cooke, 1989; Millar & Homsher, 1990; Dantzig *et al.* 1992; Potma & Stienen, 1996), which is similar to the decrease normally seen in early fatigue (Allen *et al.* 1995). However, the tetanic force in single muscle fibres and EDL muscles, as well as force at saturating $[Ca^{2+}]_i$ in the single fibres, was about 25 % lower in $CK^{-/-}$ than in the wild-type. Thus, the force reduction in fast-twitch $CK^{-/-}$ muscle appears larger than would be expected solely from the higher P_i concentration. In line with this, the absolute tetanic force after 10 fatiguing tetani was still 18 % lower in $CK^{-/-}$ than wild-type single muscle fibres. The additional force deficit in $CK^{-/-}$ fibres may be due to cytoarchitectural abnormalities within the cells and an increased fraction of the myoplasm being occupied by mitochondria and lipid droplets leading to a reduced concentration of myofibrils (Steeghs *et al.* 1997, 1998; Tullson *et al.* 1998).

Increased tetanic $[Ca^{2+}]_i$

There are several reasons why increased myoplasmic P_i might lead to increased tetanic $[Ca^{2+}]_i$. For instance, elevated P_i increases Ca^{2+}-induced SR Ca^{2+} release and the open probability of isolated SR Ca^{2+} release channels (ryanodine receptors) (Fruen *et al.* 1994; Balog *et al.* 2000). Moreover, P_i may inhibit the SR Ca^{2+} uptake (Dawson *et al.* 1980; Stienen *et al.* 1993) which, at least in the short term, might lead to increased tetanic $[Ca^{2+}]_i$ (Westerblad & Allen, 1994). Finally, P_i may decrease Ca^{2+} binding to troponin C via a reduction in strong cross-bridge attachment (Millar & Homsher, 1990) and therefore reduce the myoplasmic Ca^{2+} buffering, but this mechanism appears to be of little importance in skeletal muscle (Gordon *et al.* 2000). Thus, it appears likely that the increase of tetanic $[Ca^{2+}]_i$ seen in early fatigue (i.e. during phase 1) is due to increased P_i. This suggestion is supported by the fact that $CK^{-/-}$ muscle fibres, which do not accumulate P_i, did not display any increase of tetanic $[Ca^{2+}]_i$ during phase 1. Furthermore, the results from unfatigued fibres show a higher $[Ca^{2+}]_i$ in twitches and at the onset of 50 and 70 Hz tetani in $CK^{-/-}$ fibres. However, during the first 100 ms of 100 Hz tetani, $[Ca^{2+}]_i$ only showed a tendency (not significant) to be higher in $CK^{-/-}$ fibres. As the tetanus continued, $[Ca^{2+}]_i$ tended to decline in $CK^{-/-}$ fibres whereas it increased in wild-type fibres. This difference is exaggerated when tetani are given at short intervals, resulting in a significant transient reduction of tetanic $[Ca^{2+}]_i$ in $CK^{-/-}$ fibres (Dahlstedt *et al.* 2000). This decline of tetanic $[Ca^{2+}]_i$ in $CK^{-/-}$ fibres is probably due to a direct inhibition of the SR Ca^{2+} release channels by reduced ATP and/or increased Mg^{2+} (Blazev & Lamb, 1999), which occurs during tetanic stimulation in the absence of PCr energy buffering. In accordance with our results, this inhibition would be more marked at high stimulation frequency, where the rate of energy consumption is higher. Thus, at the onset of tetanic contraction of unfatigued $CK^{-/-}$ fibres $[Ca^{2+}]_i$ is increased due to the elevated P_i, but $[Ca^{2+}]_i$ declines as the tetanus progresses due to the lack of PCr energy buffering.

Increased myoplasmic P_i may also reduce tetanic $[Ca^{2+}]_i$ by entering the SR and precipitating with Ca^{2+}, thus reducing the Ca^{2+} available for release (Fryer *et al.* 1995; Westerblad & Allen, 1996*b*). Recent results suggest that the transport of P_i into the SR is facilitated by reduced ATP (Posterino & Fryer, 1998; Ahern & Laver, 1998). Since fast-twitch $CK^{-/-}$ muscle displays both an increased P_i and reduced ATP (Dahlstedt *et al.* 2000), some Ca^{2+}-P_i precipitation in the SR might decrease the Ca^{2+} available for release and hence reduce tetanic $[Ca^{2+}]_i$ in $CK^{-/-}$ fibres. However, this mechanism, if operating, did not prevent $CK^{-/-}$ fibres from showing a higher $[Ca^{2+}]_i$ at the onset of contractions. In this context it must be noted that the situation is markedly different in $CK^{-/-}$ fibres as compared with fibres stimulated to fatigue or injected with P_i. In the latter cases, P_i would rapidly enter the SR and precipitate with Ca^{2+} and hence reduce the Ca^{2+} available for release. In $CK^{-/-}$ fibres, on the other hand, P_i is chronically elevated and long-term homeostatic mechanisms would keep the $[Ca^{2+}]_i$ constant by adjusting the Ca^{2+} flux over the sarcolemma. Consistent with this, a decrease of resting $[Ca^{2+}]_i$ was observed with injection of P_i, which resulted in reduced tetanic $[Ca^{2+}]_i$ (Westerblad &

Allen, 1996*b*), whereas the present results did not show a lower resting $[Ca^{2+}]_i$ in $CK^{-/-}$ fibres.

Reduced myofibrillar Ca^{2+} sensitivity

The force–$[Ca^{2+}]_i$ relationship was assessed by measuring mean force and $[Ca^{2+}]_i$ over 100 ms in tetani of different frequencies. During the first 100 ms of tetanic stimulation in wild-type fibres, force and $[Ca^{2+}]_i$ reached a steady state where $[Ca^{2+}]_i$ increased slowly and this was accompanied by an increased force production. This means that the force–$[Ca^{2+}]_i$ relationship was similar whether measured 100–200 ms or 250–350 ms into the tetanus, which fits with previous results showing that the method of measuring has little impact on the force–$[Ca^{2+}]_i$ relationship (Westerblad & Allen, 1993*a*; Andrade *et al.* 1998). In $CK^{-/-}$ fibres, on the other hand, $[Ca^{2+}]_i$ tended to decline during the tetanus and this was not accompanied by a corresponding reduction of force. Hence in these fibres, Ca_{50} was about 20 % higher when measured 100–200 ms as compared with 250–350 ms into the tetanus. The results from wild-type fibres, therefore, indicate that during tetanic stimulation, force can follow slow increases of $[Ca^{2+}]_i$, i.e. additional force-producing cross-bridges are readily being formed when the Ca^{2+} activation of the thin filament increases. However, the results from $CK^{-/-}$ fibres indicate that the opposite is not true. Due to the co-operativity between strongly attached cross-bridges and thin filament activation (e.g. Swartz *et al.* 1996; Gordon *et al.* 2000), strong cross-bridges may promote new cross-bridge attachments despite lowered Ca^{2+} activation. In accordance with this, during relaxation $[Ca^{2+}]_i$ falls much faster than force. Thus, we consider the higher Ca_{50} obtained early in tetani in $CK^{-/-}$ fibres more likely to be correct.

Measurements performed 100–200 ms into tetani suggest about 15 % higher Ca_{50} in $CK^{-/-}$ fibres than in wild-type fibres. Taking data from skinned fibre experiments, a two-fold higher P_i concentration would result in an increase in Ca_{50} of 15–20 % (Millar & Homsher, 1990; Martyn & Gordon, 1992). Thus, the reduced myofibrillar Ca^{2+} sensitivity observed in $CK^{-/-}$ fibres can be explained by the higher P_i in these fibres.

Slowed relaxation

Several results of the present study support the hypothesis that the slowing of relaxation observed during early fatigue (phase 1) is due to increased P_i. First, in the unfatigued state the relaxation time was markedly longer in $CK^{-/-}$ fibres than in wild-type fibres and the former have a higher P_i concentration. Second, 10 repeated tetani increased the relaxation time in wild-type fibres (in which P_i increases during fatigue) but not in $CK^{-/-}$ fibres (in which P_i does not increase). Third, the reduced relaxation speed seen in unfatigued $CK^{-/-}$ could not be explained by a slowed Ca^{2+} removal from the myoplasm (see Fig. 3), which is similar to the results obtained in fatigued mouse fibres (Westerblad & Allen, 1993*b*). Thus, it appears that increased P_i may cause a slowing of relaxation, but the mechanism by which this occurs remains uncertain (for discussion see Westerblad *et al.* 1997; Mulligan *et al.* 1999).

In the unfatigued state, $CK^{-/-}$ EDL muscles displayed a markedly longer relaxation time than wild-type muscles, which is in agreement with the single fibre results. However, in early fatigue, EDL muscles differed from single fibres in that both tetanic force and relaxation speed was reduced in $CK^{-/-}$ muscles, albeit to a lesser extent than in wild-type muscles. This difference may be explained by some additional inhibitory factor coming into play in the whole muscle experiments and reduced intracellular pH due to lactate accumulation is a likely candidate. At the temperature used in the present study (24 °C), reduced intracellular pH will cause a marked reduction of tetanic force and relaxation speed (Westerblad *et al.* 1997). Moreover, single mouse fibres fatigue without significant acidification, probably due to effective lactate-H^+ extrusion in the absence of fatigue-induced changes of the extracellular milieu (Westerblad & Allen, 1992). In whole muscles, on the other hand, changes of metabolites in extracellular space (e.g. increased lactate concentration) will inhibit lactate-H^+ extrusion and both $CK^{-/-}$ and wild-type EDL muscles display a large increase of lactate at fatigue (Dahlstedt *et al.* 2000). Furthermore, the acidosis in early fatigue might be exaggerated in $CK^{-/-}$ muscles due to the lack of PCr hydrolysis, which consumes H^+ ions.

Slowed SR Ca^{2+} uptake

There is a marked increase in the amplitude of $[Ca^{2+}]_i$ tails after 10 fatiguing tetani (Westerblad & Allen, 1991, 1993*b*), which might be ascribed to a P_i-induced inhibition of the SR Ca^{2+} uptake. This may be due to a decreased free energy change of ATP hydrolysis (Dawson *et al.* 1980; Stienen *et al.* 1993). The present analyses of $[Ca^{2+}]_i$ after the end of tetanic stimulation are in agreement with a P_i-induced inhibition of SR Ca^{2+} uptake in early fatigue (see Fig. 4). Thus, 10 repeated tetani resulted in increased $[Ca^{2+}]_i$ tails in wild-type fibres, where PCr breaks down and P_i accumulates, but not in $CK^{-/-}$ fibres, where this does not occur.

Conclusions

The present results show that fast-twitch muscle of $CK^{-/-}$ mice provides a good model for studying the effect of P_i on muscle function. Using this model we show that increased myoplasmic P_i can explain major functional changes observed in early fatigue in single fast-twitch fibres; that is increased P_i may reduce tetanic force and myofibrillar Ca^{2+} sensitivity, increase tetanic $[Ca^{2+}]_i$, slow force relaxation, and reduce the rate of SR Ca^{2+} uptake.

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