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Contraction-mediated glycogenolysis in mouse skeletal muscle lacking creatine kinase: the role of phosphorylase b activation

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Skeletal muscle that is deficient in creatine kinase ($CK^{-/-}$) exhibits accelerated glycogenolysis during contraction. Understanding this phenomenon could provide insight into the control of glycogenolysis during contraction. Therefore, glycogen breakdown was investigated in isolated extensor digitorum longus $CK^{-/-}$ muscle. Muscles were stimulated to produce repeated tetani for 20 s in the presence of sodium cyanide to block mitochondrial respiration. Accumulation of lactate after stimulation was similar in wild-type (WT) and $CK^{-/-}$ muscles, whereas accumulation of glucose-6-phosphate was twofold higher in $CK^{-/-}$ muscles, indicating greater glycogenolysis in $CK^{-/-}$ muscles. Total phosphorylase activity was decreased by almost 30 % in $CK^{-/-}$ muscle ($P < 0.001$). Phosphorylase fractional activity ($-/+$ 3.3 mM AMP) was similar in both groups in the basal state (about 10 %), but increased to a smaller extent in $CK^{-/-}$ muscles after stimulation (39 ± 4 % *vs.* 52 ± 4 % in WT, $P < 0.05$). Inorganic phosphate, the substrate for phosphorylase, increased marginally in $CK^{-/-}$ muscles after stimulation (basal = 25.3 ± 2.2 μmol (g dry muscle)$^{-1}$; stimulated = 33.9 ± 2.3 μmol (g dry muscle)$^{-1}$), but substantially in WT muscles (basal = 11.4 ± 0.7 μmol (g dry muscle)$^{-1}$; stimulated = 54.2 ± 4.5 μmol (g dry muscle)$^{-1}$). Kinetic studies of phosphorylase b (dephosphorylated enzyme) from muscle extracts *in vitro* demonstrated higher relative activities in $CK^{-/-}$ muscles (60–135 %) in response to low AMP concentrations (up to 50 μM) in both the basal state and after stimulation ($P < 0.05$), whereas no differences in activity between $CK^{-/-}$ and WT muscles were observed at high AMP concentrations (> 100 μM). These data indicate that allosteric activation of phosphorylase b accounts for the accelerated glycogenolysis in $CK^{-/-}$ muscle during contraction.

Glycogen is the storage form of glucose in cells and is the major energy substrate that is utilized during muscle contraction. The breakdown of glycogen is catalysed by glycogen phosphorylase (EC 2.4.1.1). Phosphorylase is studied for its central role in glycogenolysis and as a model for the allosteric control of enzymes. In the reaction, a glucose residue on the nonreducing end of glycogen is phosphorylated to yield glucose 1-phosphate (glucose-1-P), which is then further metabolized in the glycolytic pathway. In addition to its role in providing a substrate during contraction, phosphorylase has recently emerged as a target for therapeutic intervention in the treatment of non-insulin-dependent diabetes mellitus (Treadway *et al.* 2001; Oikonomakos, 2002). Therefore, it is important to understand the regulation of this enzyme in living tissue.

Phosphorylase is regulated by substrate availability (inorganic phosphate, P_i), by interconversion (phos-phorylation/dephosphorylation) and allosterically, with AMP being a key activator. The phosphorylated form of phosphorylase (phosphorylase a) does not require AMP for its activity and is considered to be the active form *in vivo*. Conversely, the dephosphorylated form of phosphorylase (phosphorylase b) is fully dependent on AMP for activity and is considered to be inactive in resting muscle. Previously, it was believed that the Ca^{2+}-mediated conversion of phosphorylase b to phosphorylase a (i.e. phosphorylation of phosphorylase b) via phosphorylase b kinase was primarily responsible for the acceleration of glycogenolysis during muscle contraction (Brostrom *et al.* 1971). Subsequent studies stressed the importance of P_i availability in the activation of glycogenolysis during muscle contraction (Chasiotis *et al.* 1982, 1983), with P_i being ultimately derived from the breakdown of phospho-creatine (PCr). However, a number of studies have questioned the role of interconversion and P_i availability in

the activation of glycogenolysis during muscle contraction (Ren & Hultman, 1989; Katz, 1997). Thus the mechanism underlying the regulation of phosphorylase and glyco-genolysis in living muscle remains unclear.

Recent studies have demonstrated that skeletal muscle from mice lacking creatine kinase (CK) breaks down glycogen at a higher rate than control muscle during contraction (van Deursen *et al.* 1993, 1994). This occurs even though CK-deficient muscle has negligible increases in P_i during contraction, owing to the lack of PCr breakdown (Dahlstedt *et al.* 2000). The purpose of the study presented here, therefore, was to determine the mechanism underlying the accelerated glycogenolysis that occurs during contraction in skeletal muscle that is entirely deficient in CK ($CK^{-/-}$). The results demonstrate that neither an increase in P_i nor conversion of phosphorylase b to phosphorylase a are responsible for the accelerated glycogenolysis that occurs in $CK^{-/-}$ muscle. Rather, AMP-mediated activation of phosphorylase b appears to be a major mechanism underlying this accelerated glycogenolysis.

METHODS

Materials
α-D-[U-^{14}C]glucose-1-P and uridine diphosphate (UDP)-[U-^{14}C]glucose were from Amersham Biosciences. All other reagents and enzymes were from either Sigma or Boehringer Mannheim.

Male C57Bl/6 pure inbred mice served as wild-type (WT) controls and male $CK^{-/-}$ mice were generated as described previously (Steeghs *et al.* 1997). Animals were housed at room temperature with a 12 h:12 h light:dark cycle. Food and water were provided *ad libitum*. Animals were killed by rapid cervical dislocation, and thereafter the extensor digitorum longus (EDL) muscles were isolated. The local Stockholm North ethical committee approved all procedures.

Muscle mounting, solutions and stimulation
Stainless-steel hooks were tied with nylon thread to the tendons of the muscle. Muscles were then transferred to a stimulation chamber and mounted between a laboratory-built force trans-ducer and an adjustable holder. The chamber contained a Tyrode solution of the following composition (mM): NaCl 121, KCl 5, $CaCl_2$ 1.8, NaH_2PO_4 0.5, $MgCl_2$ 0.4, $NaHCO_3$ 24, EDTA 0.1, glucose 5.5 and 0.1% fetal calf serum. The solution was gassed continuously with 5%CO_2/95% O_2, which results in a pH of 7.4. After setting the optimal length with twitches, muscles were allowed to recover for 30 min, Sodium cyanide (NaCN) was added to the solution in the chamber 25 min after setting the optimal length, yielding a final concentration of 3 mM. At 30 min muscles were either frozen in liquid N_2 (non-stimulated) or stimulated with current pulses (0.5 ms duration; ~150% of the voltage required for a maximum force response) for 330 ms at 70 Hz via platinum plate electrodes placed parallel to the fibres. Tetani were administered at intervals of 1 s^{-1} for 10 or 20 s. After the final tetanus, the muscle was removed from the chamber and frozen, with a delay between removal and freezing of about 5 s.

Analyses
Force signals were sampled on-line and stored in a desktop computer for subsequent analysis. The muscles were stored in

liquid N_2 until they were freeze-dried. The hooks were removed and the muscles were dissected free of non-muscle constituents and then powdered. The powder was thoroughly mixed and aliquoted.

For analyses of phosphorylase and glycogen synthase (GS), incorporation of label from [^{14}C]glucose-1-P and UDP-[^{14}C]-glucose into glycogen were determined with filter paper techniques (Thomas *et al.* 1968; Tan & Nuttall, 1975), as described previously (Jiao *et al.* 1999), with slight modifications. Briefly, muscle powder was homogenized in ice-cold buffer (200 μl (mg dry muscle)$^{-1}$) containing 30% (v/v) glycerol, 10 mM EDTA and 50 mM potassium fluoride (KF), pH 7.0, with a ground-glass homogenizer, and centrifuged at 10 000 g for 20 min at 4 °C. The supernatant was then divided into two aliquots. One aliquot was diluted (2:1) with an ice-cold buffer containing 100 mM Mes and 100 mM KF, pH 6.3, and assayed for phosphorylase in the direction of glycogen synthesis. Phosphorylase a was assayed in the absence of AMP and in the presence of 60 mM glucose-1-P (22 500 dpm μmol^{-1}), and 6.7 (mg glycogen) ml^{-1} at pH 6.3 for 20 min at 30 °C. Phosphorylase was also assayed in the presence of various concentrations of AMP up to 3.3 mM. Phosphorylase b activity was considered to be the difference between the activity measured in the absence and in the presence of AMP. It might be argued that AMP-dependent activity does not solely reflect the activation of phosphorylase b, since AMP also activates phosphorylase a (Lowry *et al.* 1964; Aragon *et al.* 1980). However, at the high concentrations of glucose-1-P and glycogen used, the effect of AMP on phosphorylase a will be negligible (Lowry *et al.* 1964). Phosphorylase activity is expressed as micromoles of glucose from glucose-1-P incorporated into glycogen per minute per gram of dry muscle. Phosphorylase fractional activity was defined as the ratio between the activities measured in the absence of AMP and presence of 3.3 mM AMP (total phosphorylase). Endogenous AMP in the assay mixture could be estimated to be about 0.1 μM, which would not interfere with our measurements (see Results).

The other aliquot of supernatant was diluted (1:4) with an ice-cold buffer containing 50 mM Tris, 20 mM EDTA and 130 mM KF, pH 7.8, and assayed for GS at a low glucose-6-phosphate (glucose-6-P) concentration (0.17 mM) and a high glucose-6-P concentration (7.2 mM, GS_{high}) using 0.13 mM UDP-glucose (5000 dpm nmol^{-1}) at pH 7.8 and 30 °C for 10 min (Guinovart *et al.* 1979). Activity is expressed as micromoles of glucose from UDP-glucose incorporated into glycogen per minute per gram of dry muscle. GS fractional activity (GSF) is the ratio of the activity measured in the presence of 0.17 and 7.2 mM glucose-6-P.

For analysis of glycogen, aliquots of powder were digested with hot, 1 N KOH and hydrolysed enzymatically to free glucose (Jiao *et al.* 1999). Glucose was then analysed enzymatically with the aid of a fluorometric technique (Lowry & Passonneau, 1972). For analysis of metabolites, ice-cold 0.5 M perchloric acid was added to aliquots of muscle powder. The extract was kept in an ice bath for 15 min while being agitated with a vortex mixer, and then centrifuged (10 000 g). The supernatant was neutralized with 2.2 M $KHCO_3$ and then centrifuged again. The latter supernatant was assayed for PCr, creatine (Cr), ATP, P_i, glucose-6-P and lactate, with the aid of enzymatic techniques (changes in NAD[P]H) adapted for fluorometry (Lowry & Passonneau, 1972). High-performance liquid chromatography was used to analyse the extracts for inosine monophosphate (IMP), inosine, ADP and AMP (Norman *et al.* 1991). To adjust for variability in solid nonmuscle constituents, metabolite values were divided by

Table 1. Glycogenolytic intermediates in EDL muscle

	Basal		Stimulated	
	WT	CK$^{-/-}$	WT	CK$^{-/-}$
Glycogen	47 ± 5	107 ± 9***	n.d.	n.d.
Glucose-6-P	0.4 ± 0.1	1.7 ± 0.3***	1.7 ± 0.4	4.5 ± 0.6**
Lactate	1.8 ± 0.9	3.2 ± 1.4	27.5 ± 4.1	23.3 ± 2.2

Values are means ± S.E.M. for 5–10 muscles and are given in micromoles per gram of dry muscle, or for glycogen in micromoles of glucosyl units per gram of dry muscle. Isolated EDL muscles were incubated in oxygenated Tyrode (basal) or stimulated in oxygenated Tyrode containing 3 mM NaCN for 20 s and immediately frozen in liquid nitrogen. n.d., not determined. **$P < 0.01$; ***$P < 0.001$ *vs.* WT.

the sum of PCr + Cr (total Cr) and then multiplied by the mean total Cr content for the whole material (83.8 ± 2.2 μmol (g dry muscle)$^{-1}$).

Statistical analysis

Values are given as means ± S.E.M., unless indicated otherwise. Statistically significant differences ($P < 0.05$) were determined with the unpaired *t* test.

RESULTS

Glycogenolysis, phosphorylase and metabolites

Pilot experiments were performed to determine the optimal conditions of study. Muscles were stimulated for 10 or 20 s. Peak increases in phosphorylase fractional activity (PF) were achieved at 10 s and did not change further at 20 s in both groups (data not shown). Later time points were not studied because of the likelihood of decreases in PF during further stimulation, as well as decreases in the rate of glycogenolysis (Danforth & Lyon, 1964; Piras & Staneloni, 1969; Staneloni & Piras, 1969). Twenty seconds was chosen as the time point of study to: (1) allow for sufficiently large changes in glycogenolytic intermediates to be measured, (2) ensure a high and fairly linear glycogenolytic rate (Staneloni & Piras, 1969) and (3) ensure large increases in PF. Moreover, the experiments were performed in the presence of 3 mM NaCN to inhibit mitochondrial respiration (Sahlin & Katz, 1986). During 20 s of repeated tetani, there was no change in the lactate concentration in the medium (data not shown). Thus, increases in glycogenolytic intermediates under conditions where there is no oxidative metabolism nor release of such intermediates (i.e. lactate) from the muscle reflect the extent of glycogenolysis (Harris *et al.* 1981; Sahlin *et al.* 1989). This approach is superior to direct measurements of glycogen for estimating glycogenoylsis under conditions where the absolute change in glycogen is relatively small (Harris *et al.* 1975; Katz & Raz, 1995; Katz, 1997).

Glycogen content in the basal state was more than twofold greater in CK$^{-/-}$ muscle (Table 1). Lactate contents were similar between groups in the basal state and after stimulation, suggesting similar glycolytic (post-phospho-fructokinase) flux. Glucose-6-P was significantly higher in the basal state and after stimulation in CK$^{-/-}$ muscle. The increase in glucose-6-P during stimulation was more than twofold greater in CK$^{-/-}$ muscle. As discussed elsewhere (Katz *et al.* 1986*a*; Katz & Sahlin, 1990), during short-term, intense muscle contraction, the phosphorylation of extra-cellularly derived glucose to glucose-6-P by hexokinase is negligible. Therefore, the larger glucose-6-P accumulation (together with the similar lactate accumulation) indicates increased glycogen breakdown in CK$^{-/-}$ muscle during the 20 s of repeated tetani, which is consistent with earlier reports based on direct measurements of glycogen during more prolonged stimulations (van Deursen *et al.* 1993, 1994).

Twenty seconds of stimulation in the presence of NaCN resulted in PF values that were not significantly different than those obtained in the absence of NaCN for both CK$^{-/-}$ and WT muscles (data not shown). Therefore, the PF results from the two series of experiments were pooled.

Figure 1. Phosphorylase activation is blunted in CK$^{-/-}$ muscle during contraction

EDL muscles from CK$^{-/-}$ (○) and WT (●) mice were either not simulated (0 s) or stimulated with 330 ms tetani (1 s^{-1}) for 10 or 20 s in the absence or presence of 3 mM NaCN. Since NaCN did not significantly affect the results, they have been pooled. The 10 s stimulations were performed only in the absence of NaCN. Phosphorylase was measured in the absence and presence of 3.3 mM AMP and the fractional activity is the ratio of the two. Values are means ± S.E.M. for 8–18 muscles, except for WT at 10 s, where $n = 2$. CK$^{-/-}$ values during contraction are significantly lower than for WT ($P < 0.05$).
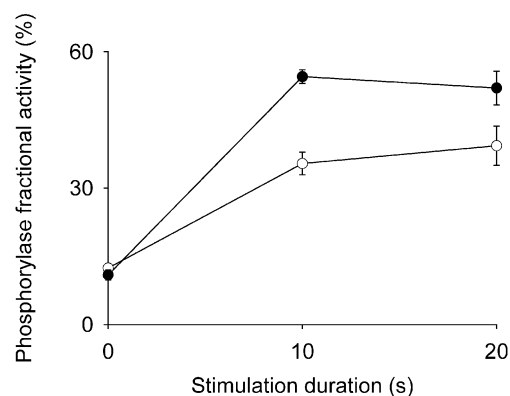
The increase of PF during contraction was blunted in $CK^{-/-}$ muscle (Fig. 1). This blunting occurred despite the observation that during repeated tetani, increases in myoplasmic Ca^{2+}, which activates phosphorylase kinase, are comparable in $CK^{-/-}$ and WT muscle (Dahlstedt *et al.* 2000; Bruton *et al.* 2003). Total phosphorylase activity (+3.3 mM AMP) was about 30 % lower in $CK^{-/-}$ muscle (175 ± 4 μmol min^{-1} (g dry muscle)$^{-1}$, $n = 31$, *vs.* 240 ± 7 μmol min^{-1} (g dry muscle)$^{-1}$ in WT muscle, $n = 35$; $P < 0.001$). Thus, glycogen breakdown was higher despite the fact that both total phosphorylase and PF were lower during stimulation in $CK^{-/-}$ muscle. Indeed, the activity of phosphorylase a in $CK^{-/-}$ muscle during contraction was only about half of that in WT muscle (69 *vs.* 125 μmol min^{-1} (g dry muscle)$^{-1}$).

The contents of high-energy phosphates and catabolites of ATP degradation are given in Table 2. As shown previously, levels of PCr did not decrease during contraction in $CK^{-/-}$ muscle, whereas large decreases occurred in WT muscle (cf. Dahlstedt *et al.* 2000). Surprisingly, the decrease in ATP was substantially smaller during stimulation in $CK^{-/-}$ muscle. Total muscle AMP content was significantly higher in $CK^{-/-}$ muscle in the basal state, but the difference was not significantly different after stimulation. The

increase in IMP was stoichiometric to the decrease in ATP in both groups. The P_i content was significantly higher in $CK^{-/-}$ muscle in the basal state, as shown previously (Dahlstedt *et al.* 2000). Of particular importance is that there was little increase in P_i during stimulation in $CK^{-/-}$ muscle compared with a fivefold increase in WT muscle. Whereas the P_i content was higher at the onset of contraction in $CK^{-/-}$ muscle, it is clear that within a few seconds of contraction, when the rate of PCr degradation is highest (Hultman & Sjoholm, 1983), the P_i content rose to higher levels in WT muscle. Hence, the P_i content was probably higher during most of the 20 s contraction period in WT muscle. Thus, $CK^{-/-}$ muscle exhibited a higher glycogenolytic rate during contraction, despite a lower P_i content.

Since neither interconversion (phosphorylation) nor substrate availability (P_i) could account for the higher glycogen breakdown in $CK^{-/-}$ muscle during contraction, we reasoned that activation of phosphorylase b would explain the results. A major allosteric activator of phosphorylase b is AMP. Therefore the activity of phosphorylase was measured in muscle extracts in the presence of increasing concentrations of AMP (Fig. 2). In the presence of low concentrations of AMP ($\leqslant 100$ μM), a
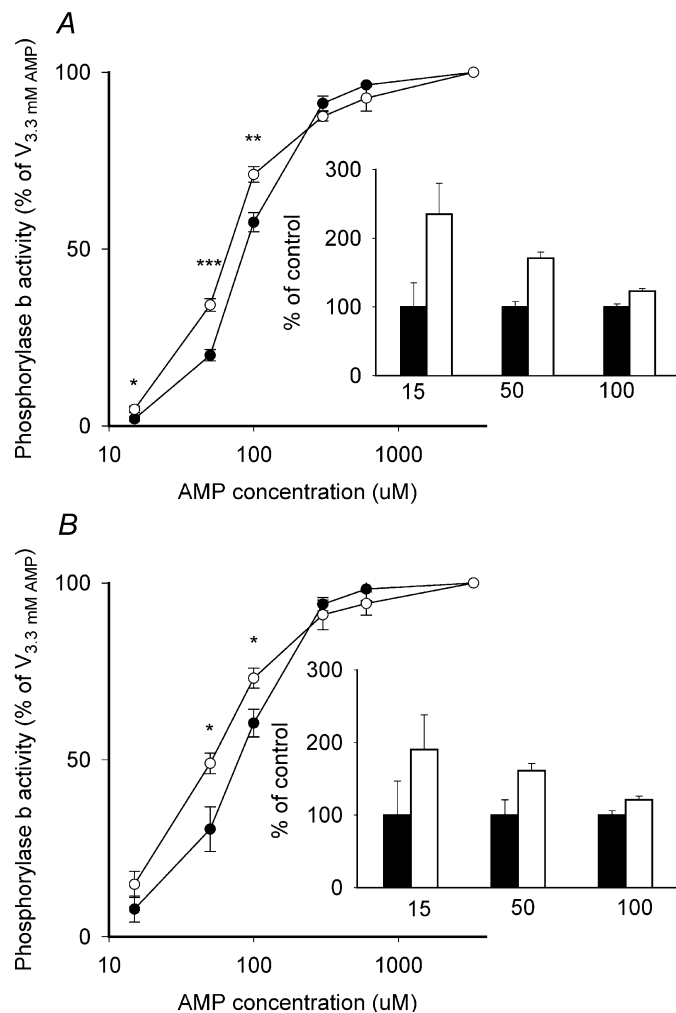

Figure 2. Phosphorylase b sensitivity to AMP is increased in extracts from $CK^{-/-}$ muscle

Extracts were prepared from $CK^{-/-}$ (○) and WT (●) EDL muscles that were either not stimulated (*A*) or stimulated for 20 s (*B*) and analysed for phosphorylase activity with various concentrations of AMP, as described in the text. Activity in the absence of AMP was set to zero and the activity measured in the presence of AMP is considered to reflect phosphorylase b activity. Insets show activities at low AMP concentrations, where mean WT values (control, black bars) are set to 100 % and $CK^{-/-}$ values (open bars) are given as a percentage of WT values. Values are means ± s.e.m. for 6–8 muscles. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ between WT and $CK^{-/-}$ at a given AMP concentration.

Table 2. High-energy phosphate metabolism in EDL muscle

	Basal		Stimulated	
	WT	CK$^{-/-}$	WT	CK$^{-/-}$
Phosphocreatine	66.3 ± 1.4	44.6 ± 0.9***	24.4 ± 3.9	45.7 ± 2.3***
Creatine	17.7 ± 1.4	39.3 ± 1.0***	59.6 ± 3.9	38.4 ± 2.3 ***
P$_i$	11.4 ± 0.7	25.3 ± 2.2***	54.2 ± 4.5	33.9 ± 2.3***
ATP	22.6 ± 0.5	16.7 ± 0.3***	15.3 ± 1.4	14.0 ± 1.4
ADP	2.8 ± 0.1	3.6 ± 0.2**	3.1 ± 0.1	4.0 ± 0.3*
AMP	0.09 ± 0.01	0.16 ± 0.01**	0.11 ± 0.02	0.16 ± 0.03
IMP	0.37 ± 0.10	0.28 ± 0.06	8.2 ± 1.2	2.5 ± 0.6**
Inosine	0.09 ± 0.05	0.50 ± 0.18**	0.15 ± 0.10	0.77 ± 0.25*
ATP turnover	—	—	4.8	1.7
Force–time integral	—	—	483 ± 67	201 ± 48**
Mechanical efficiency	—	—	101	118

Values are means (± S.E.M., when given) for 4–10 muscles and are given in micromoles per gram of dry muscle for metabolites. Isolated EDL muscles were incubated in oxygenated Tyrode (basal) or stimulated in oxygenated Tyrode containing 3 mM NaCN for 20 s and immediately frozen in liquid nitrogen. ATP turnover during the 20 s is calculated from mean values as follows: $(-2\Delta\text{ATP} - \Delta\text{ADP} - \Delta\text{phosphocreatine} + 1.5\Delta\text{lactate})/20\,\text{s}$ and given in micromoles per second per gram of dry muscle. The force–time integral is given in $(\text{N} \times \text{s})/(\text{g dry muscle})$. Mechanical efficiency is calculated as mean force–time integral divided by ATP turnover and is expressed as $(\text{N} \times \text{s}^2)/\mu\text{mol}$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ *vs.* WT.

greater proportion of phosphorylase b was activated in the CK$^{-/-}$ muscle both in the basal state (Fig. 2, top) and after stimulation (Fig. 2, bottom). At concentrations of up to 50 μM AMP, the relative activation of phosphorylase b was on average 60–135 % higher in CK$^{-/-}$ *vs.* WT muscle (insets of Fig. 2). At both 15 and 50 μM AMP, a greater proportion of phosphorylase b was activated after contraction *vs.* basal in the CK$^{-/-}$ muscle (15 μM AMP: basal = 4.7 ± 0.9 %, stimulated = 14.8 ± 3.7 %, $P < 0.05$; 50 μM AMP: basal = 34.2 ± 1.8 %, stimulated = 49.0 ± 2.9 %, $P < 0.01$). No significant contraction-induced changes were noted in the WT muscle extracts (Fig. 2).

Glycogen synthase

Because glycogen content is a function of both breakdown and synthesis, we also measured the activity of GS. GS$_{high}$ in the basal state was increased by about 60 % in CK$^{-/-}$ muscle (6.4 ± 0.3 μmol min^{-1} (g dry muscle)$^{-1}$ ($n = 11$) *vs.* 4.0 ± 0.4 μmol min^{-1} (g dry muscle)$^{-1}$ in WT ($n = 9$), $P < 0.001$) and no significant changes occurred during contraction in

either group (data not shown). GSF averaged 0.37 ± 0.03 ($n = 9$) in WT in the basal state and decreased to 0.24 ± 0.03 ($n = 8$) after 20 s of contraction ($P < 0.01$). GSF averaged 0.20 ± 0.02 ($n = 11$) in CK$^{-/-}$ in the basal state ($P < 0.001$ *vs.* WT) and did not change after 20 s of contraction (0.21 ± 0.04, $n = 6$, $P > 0.05$). It is possible that the elevated GS$_{high}$ activity and glucose-6-P (activates GS) content contributed to the increased glycogen level in the CK$^{-/-}$ muscle in the basal state. Furthermore, the glucose-6-P content after stimulation in both groups (equivalent to 0.9 and 2.3 mM for WT and CK$^{-/-}$, respectively) would indicate that GS was almost fully active, assuming activation constant (K_a) values of GS for glucose-6-P of 0.2–0.5 mM (Larner & Villar-Palasi, 1971). Nevertheless, assuming a K_m value of GS for UDP-glucose of 0.5 mM (Larner & Villar-Palasi, 1971) and a UDP-glucose concentration of 15 μM in stimulated mouse muscle (Passonneau *et al.* 1969), the rate of glycogen formation will be negligible compared with the rate of breakdown during contraction.

Figure 3. Fatigue is accelerated in CK$^{-/-}$ muscle during repeated contractions in the presence of cyanide

EDL muscles were stimulated with 330 ms tetani at 1 s intervals. Values are means ± S.E.M. for CK$^{-/-}$ ($n = 6$, unfilled circles) or WT ($n = 8$, filled circles) muscles. Other than the first tetanus, differences between groups are significant during all contractions ($P < 0.01$–0.001).
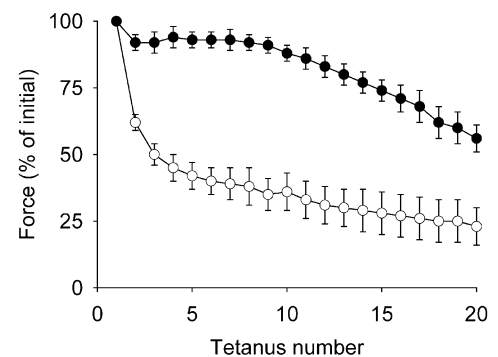
Force and fatigue

Initial force was slightly, but not significantly lower in CK$^{-/-}$ muscles (68 ± 16 N (g dry muscle)$^{-1}$ *vs.* 82 ± 12 N (g dry muscle)$^{-1}$ in WT). However, CK$^{-/-}$ muscles fatigued faster and to a greater extent during stimulation (Fig. 3). On average, force decreased to 92 ± 3 % of initial in WT and 62 ± 3 % CK$^{-/-}$ muscles during the second tetanus ($P < 0.001$) and to 56 ± 5 % and 23 ± 7 % ($P < 0.01$), respectively, during the last tetanus. The force–time integral in CK$^{-/-}$ muscle amounted to only about 40 % of that in WT muscle (Table 2). Interestingly, adjusting glycogenolysis for differences in force production ((Δglucose-6-P + 0.5Δlactate)/(force–time integral)) yields a glycogenolytic rate in CK$^{-/-}$ muscle that is more than twofold higher than in WT muscle. The mechanical efficiency ((force–time integral)/ATP turnover) was increased by about 20 % in CK$^{-/-}$ muscles during contraction (Table 2).

DISCUSSION

An understanding of the regulation of glycogen break-down during muscle contraction has remained elusive. The roles of increased PF and availability of P$_i$ have been questioned in terms of explaining high glycogenolytic rates in skeletal muscle, since ischaemic, noncontracting muscle, despite elevated PF (i.e. phosphorylase a) and P$_i$ values, exhibits negligible glycogenolysis (Ren & Hultman, 1989; Katz, 1997). If the same PF and Pi contents are obtained during muscle contraction, then the glycogenolytic rate is about 250-fold higher than during ischemia (Katz, 1997). The results of the present study demonstrate that high glycogenolytic rates occur in CK$^{-/-}$ muscle, despite low PF and P$_i$ values.

Glycogenolysis and the allosteric activation of phosphorylase

How, then, can one explain the high glycogenolytic rate in CK$^{-/-}$ muscle? It could be argued that the higher basal glycogen content could increase glycogenolysis in CK$^{-/-}$ muscle during contraction, as has been suggested by some (Hespel & Richter, 1992). And, although the K_m of phosphorylase for glycogen *in vitro* is about 2 mM (Brown & Cori, 1961), it has been suggested that the value may be higher in living muscle (Richter & Galbo, 1986). However, most studies performed during high-intensity, short-term exercise indicate that the rate of glycogenolysis is independent of the initial glycogen level (Sahlin *et al.* 1989; Spriet *et al.* 1990; Ren *et al.* 1990; Spencer & Katz, 1991). This speaks against the idea that the higher glycogen content in CK$^{-/-}$ muscle is responsible for the higher rate of glycogen breakdown under the conditions studied. Therefore, the most likely explanation for the high glycogenolytic rate in CK$^{-/-}$ muscle is the allosteric activation of phosphorylase.

It has been suggested that the major allosteric activator of phosphorylase during contraction is the transient increase in AMP concentration at the enzymatic site (Katz & Sahlin, 1990). The relevant species of AMP (free concentration at the enzymatic site during the contraction) can be neither measured directly nor calculated, for reasons that are discussed elsewhere (Katz *et al.* 1986*b*; Katz & Sahlin, 1990; Sahlin, 1991). However, AMP is a major determinant of flux through AMP deaminase at high rates of energy turnover (Katz *et al.* 1986*b*; Sahlin *et al.* 1990). Under these conditions, AMP is deaminated by AMP deaminase to IMP and NH$_3$, and the removal of IMP is a very slow process that occurs during recovery from contraction (Meyer & Terjung, 1980). Thus, increases in IMP in frozen biopsy samples have been used as an indirect measure of AMP transients during the contraction phase (Broberg *et al.* 1988; Katz & Sahlin, 1990). Based on the observation of a greater decrease in ATP in CK$^{-/-}$ muscle during ischaemia (in 't Zandt *et al.* 1999), we predicted that the decrease in ATP and increase in IMP would be larger in CK$^{-/-}$ muscle during intense anaerobic contractions. We assumed that owing to the lack of CK activity, coupled with a high rate of ATP hydrolysis, the ADP transients would be elevated in CK$^{-/-}$ muscle. In addition, in the presence of normal levels of AK in CK$^{-/-}$ muscle (Ventura-Clapier *et al.* 1995), the excess ADP would be converted to AMP. Indeed, mathematical modelling of intracellular concentrations of free ADP and AMP during contraction indicate markedly larger increases in these nucleotides in muscles devoid of cytosolic CK (Roman *et al.* 2002). This would have allowed for the conclusion that the high level of glycogenolysis in CK$^{-/-}$ muscle could be attributed to larger AMP transients, which could activate both phosphorylases a and b. Surprisingly, the ATP depletion and IMP accumulation in CK$^{-/-}$ muscle after contraction were decreased to about 25 % of those seen in WT muscle. It should be noted, however, that AMP deaminase activity is decreased in the fast-twitch muscle of CK$^{-/-}$ mice also to about 25 % of that in WT muscle (Tullson *et al.* 1998). This could account for the observed decrease in AMP deamination in CK$^{-/-}$ muscle. Thus, larger AMP transients could explain the high glycogenolytic rate in CK$^{-/-}$ muscle by activating both phosphorylases a and b (Lowry *et al.* 1964).

A novel finding of the present study was that the phosphorylase b from CK$^{-/-}$ muscle exhibited an increased affinity for AMP in both the basal state and after contraction. This observation raises the possibility that the AMP-mediated activation of phosphorylase b contributes to the accelerated glycogenolysis in CK$^{-/-}$ muscle. Consistent with this idea is the observation that the average rate of glycogenolysis during the contraction (which amounted to 6.6 s of the 20 s) is about 120 μmol min^{-1} (g dry muscle)$^{-1}$ (accumulation of 13 μmol of

glycogenolytic intermediates (Δglucose-6-P + 0.5Δlactate from Table 1) per 6.6 s). This activity is almost twice that of the phosphorylase a activity measured in the CK$^{-/-}$ muscle extracts after contraction, indicating that the measured phosphorylase a activity was insufficient to account for the observed rate of glycogen breakdown. Noteworthy is that we are underestimating glycogenolysis by about 10 % (lacking other hexose phosphates and glycolytic intermediates; Katz *et al.* 1986*c*) and we are overestimating the 'physiological' phosphorylase activity, which is usually higher when measured in the direction of glycogen synthesis (see Danforth & Lyon, 1964). Thus the discrepancy between the measured glycogen breakdown and the measured *in vitro* activity of phosphorylase a is likely to be greater than we estimate.

One can question whether the free AMP concentration in the myoplasm can reach the levels required to substantially activate phosphorylase b during contraction, since it is generally believed that this concentration is very low (< 1 μM; Roman *et al.* 2002). Interestingly, it has been estimated by independent means that in muscles in which PCr breakdown is compromised (e.g. during fatigue), free ADP concentrations can reach values exceeding 1 mM (Westerblad *et al.* 1998; Roman *et al.* 2002). If these values are correct, then one can estimate the AMP concentration via the AK reaction ([AMP] = K_{AK} × [ADP]^2/[ATP], where K_{AK} = 1.0; Roman *et al.* 2002). Assuming respective ADP and ATP values of 1 and 7 mM (see Table 2; and see Dahlstedt *et al.* (2001) for conversion to mM), the myoplasmic AMP concentration can be estimated to be almost 0.15 mM. This would be sufficient to activate phosphorylase b to > 50 % of V_{max}.

At present, the mechanism underlying the increased affinity of phosphorylase b from CK$^{-/-}$ muscle for AMP in the basal state, as well as the further increase in affinity in stimulated muscle is unclear. Indeed, we also do not know whether the same mechanism is responsible for the increased affinity observed under both conditions. It is possible that alterations in free thiol availability induced by reactive oxygen species (Ji *et al.* 1988) or novel covalent modifications (e.g. phosphorylation, acetylation and glucosylation) are involved. This remains a matter for future investigation.

CK$^{-/-}$ muscles and fatigue

Previously, it was shown that EDL CK$^{-/-}$ muscles were slightly more fatigue resistant than WT muscles during repeated tetanic contractions (Dahlstedt *et al.* 2000). However, in the present study, force decreased rapidly in the second and third tetani in CK$^{-/-}$ muscles and thereafter continued to decrease at a slower rate, whereas the force decrease was markedly smaller in WT muscles. The difference in fatigue resistance between studies can be attributed to the NaCN-mediated inhibition of mito-

chondrial respiration (Bruton *et al.* 2003; Dzeja *et al.* 2003). The rapid force decline in CK$^{-/-}$ muscles in the present study is likely to be due to metabolic changes occurring as a consequence of the inability to rephosphorylate ADP via CK. The increased ADP concentration (see above) will reduce the rate of crossbridge cycling, while having little effect on the ability to generate force (Cooke & Pate, 1985). Thus, increased levels of ADP may explain the decreased ATP turnover and the increased mechanical efficiency in CK$^{-/-}$ muscles.

Conclusions

The results of this study demonstrate that the high rate of glycogen breakdown that occurs in CK$^{-/-}$ muscle during repeated contractions cannot be attributed to the conversion of phosphorylase b to phosphorylase a, nor to increases in the concentration of P$_i$. It is suggested that it is AMP-mediated activation of phosphorylase b that is primarily responsible for the accelerated glycogen breakdown. The activation can occur by increases in the concentration of the relevant species of AMP (transients at the enzymatic site during contraction) and by increasing the affinity of phosphorylase b for AMP.

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