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Combined myofibrillar and mitochondrial creatine kinase deficiency impairs mouse diaphragm isotonic function

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Watchko, Jon F., Monica J. Daood, Gary C. Sieck, John J. LaBella, Bill T. Ameredes, Alan P. Koretsky, and Be Wieringa. Combined myofibrillar and mitochondrial creatine kinase deficiency impairs mouse diaphragm isotonic function. *J. Appl. Physiol.* 82(5): 1416–1423, 1997.—Creatine kinase (CK) is an enzyme central to cellular high-energy phosphate metabolism in muscle. To characterize the physiological role of CK in respiratory muscle during dynamic contractions, we compared the force-velocity relationships, power, and work output characteristics of the diaphragm (Dia) from mice with combined myofibrillar and sarcomeric mitochondrial CK deficiency (CK[–/–]) with CK-sufficient controls (Ctl). Maximum velocity of shortening was significantly lower in CK[–/–] Dia ($14.1 \pm 0.9 L_0/s$, where L_0 is optimal fiber length) compared with Ctl Dia ($17.5 \pm 1.1 L_0/s$) ($P < 0.01$). Maximum power was obtained at 0.4–0.5 tetanic force in both groups; absolute maximum power ($2,293 \pm 138 W/m^2$) and work ($201 \pm 9 J/m^2$) were lower in CK[–/–] Dia compared with Ctl Dia ($2,744 \pm 146 W/m^2$ and $284 \pm 26 J/m^2$, respectively) ($P < 0.05$). The ability of CK[–/–] Dia to sustain shortening during repetitive isotonic activation (75 Hz, 330-ms duration repeated each second at 0.4 tetanic force load) was markedly impaired, with CK[–/–] Dia power and work declining to zero by 37 ± 4 s, compared with 61 ± 5 s in Ctl Dia. We conclude that combined myofibrillar and sarcomeric mitochondrial CK deficiency profoundly impairs Dia power and work output, underscoring the functional importance of CK during dynamic contractions in skeletal muscle.

respiratory muscle; fatigue; myosin heavy chain

RESPIRATORY MUSCLE FATIGUE is a phenomenon of clinical importance contributing to the development of ventilatory failure in neonates and adults. The factors predisposing muscle to fatigue are felt primarily to result from an imbalance between energy supply and demand (25, 35). The accumulation of energy metabolites in all probability also plays an important role (1, 6, 8). Alteration of the capacity of skeletal muscle to produce or sustain ATP levels is thus likely to affect muscle fatigue resistance. Creatine kinase (CK), an enzyme central to cellular high-energy phosphate metabolism in muscle, may be important in this regard. CK catalyzes the following reaction



where PCr is phosphocreatine and Cr is creatine. The high level of CK activity found in skeletal muscle ensures that, when consistent high-energy phosphate production is necessary during repetitive contractile activity, ATP levels will be maintained at the expense of PCr. This role of CK as an ATP buffer is widely accepted (18, 33).

Defining a physiological role for CK in skeletal muscle fatigue, however, has been difficult, with prior studies using substrate analogs (10, 16, 17, 20) of Cr to assess the functional consequences of impaired CK activity. These studies have generated disparate results and exclusively examined fatigue under repetitive isometric conditions. The respiratory muscles' capacity to sustain ventilation, however, is determined in large part by its ability to shorten and maintain force during repetitive dynamic contractions (3, 22). Moreover, dynamic contractions are known to have higher energetic demands (9, 12) than those of an isometric nature that should further challenge the CK-PCr system.

In the present study, we took an alternative approach to ascertain the role of CK in respiratory muscle function by using reverse genetics to alter CK expression in the intact animal. Specifically, we studied the diaphragm muscle (Dia) from mice bearing combined null mutations for the sarcomeric mitochondrial CK (ScCKmit) (27, 28) and myofibrillar CK (M-CK) genes (32) and determined the effect of this blockade in the CK system on respiratory muscle function during repetitive isotonic activation. These ScCKmit/M-CK double-deficient mutant (CK[–/–]) mice have recently been generated by using targeted mutagenesis (27). We hypothesized that CK[–/–] double-deficient Dia would have a marked impairment in shortening capacity, as indexed by decrements in the extent of muscle shortening, muscle shortening velocity, power, and work during repetitive isotonic activation, as compared with wild-type CK-sufficient Dia. Because CK isoforms are structurally associated with sites of energy consumption [M-CK and actomyosin adenosinetriphosphatase (ATPase) on the myosin heavy chain (MHC)] (33) and isotonic contractile properties are related to myosin isoform composition, we also determined the effect of CK deficiency on MHC phenotype.

METHODS

Mice bearing a null mutation of the ScCKmit $[-/-]$ gene (27, 28) were interbred with mice bearing a null mutation of the M-CK gene (32) to generate heterozygotes for both CK isoforms. Sibling matings of these mice resulted in the generation of ScCKmit/M-CK double-deficient mutant CK $[-/-]$ mice, as previously described and confirmed by polymerase chain reaction analysis (27). Control and ScCKmit/M-CK double-deficient mice had a mixed genetic (C57B1/6 \times 129/Sv) background. Eleven control (Ctl) and 11 ScCKmit/M-CK double-deficient adult (90–110 days) mice were used in the study, and the experiments were approved by the Magee-Womens Hospital Institutional Animal Care and Use Committee. Animals were anesthetized with pentobarbital sodium (60 mg/kg ip), and individual segments of diaphragm were excised for 1) *in vitro* assessment of isometric and isotonic contractile properties, 2) measurement of CK activity, and 3) determination of MHC phenotype.

In vitro measurements. Dia muscle strips (~2 mm wide) were cut from the midcostal region of the right hemidiaphragm, with fiber attachments at the rib and central tendon left intact. The muscle segments were mounted in a vertical tissue chamber, which was constantly perfused with mammalian Ringer solution aerated with 95%O₂-5%CO₂ and maintained at 37°C. The monitored PO₂, PCO₂, and pH were 400–460 Torr, 35–40 Torr, and 7.35–7.40, respectively. The costal margin origin of fibers was fixed by use of a vascular clamp mounted in series to a micropositioner near the base of the tissue chamber. A small piece of aluminum foil was glued to the central tendon with cyanoacrylate and then attached to the force transducer (model 300B, Cambridge Technology) via fine wire. This provided a noncompliant attachment to the force transducer and prevented tearing of the central tendon. The muscle bundles were stimulated directly (Grass model S-88 stimulator and current amplifier) by use of monophasic rectangular pulses of cathodal current (1.0-ms duration) delivered through platinum plate electrodes placed ~1 cm apart. Muscle bundles were positioned midway between the two electrodes. To ensure supramaximal stimulation, current was increased by 50% over the current necessary to obtain peak twitch force (~250–300 mA). Muscle fiber length was adjusted incrementally by using a micropositioner until maximal isometric twitch force (P_t) responses were obtained [i.e., optimal fiber length (L_o)]. Twitch contraction (CT) and half relaxation ($RT_{1/2}$) times were also determined. Maximum tetanic force (P_o) was assessed by stimulating the muscle bundle at 75 Hz delivered in a 1-s train. Force and length signals of the Cambridge dual-mode servo-control module were displayed on a digital oscilloscope (model 1602, Gould), digitized at 500 Hz, and stored on a computer disk file. The stimulation paradigm and isotonic afterloaded contractions were controlled by a computer program (LabView 3.1, National Instruments).

Shortening velocities were measured at eight different loads (5–50% of P_o) during isotonic afterloaded contractions. Velocities were calculated by computer from the maximum slope of the digitized length signal in the interval between 10 and 30 ms after the beginning of the isotonic shortening phase. Loads were calculated as a fraction of P_o based on the force plateau measured during the isotonic contraction. The data were fitted by a modified version (2) of Hill's hyperbolic equation $[(V + b)(P/P_o + a/P_o) = b(1 + a/P_o)]$ (12) by using a least squares technique, and maximum velocity of shortening (V_{max}) was calculated from the optimum a/P_o and b values as $V_{max} = b/(a/P_o)$. V_{max} is reported in L_o per second. Power was calculated as the product of the isotonic afterload and velocity

of shortening and expressed in watts per square meter. Work was calculated as the product of the isotonic afterload and extent of lengthening and expressed in joules per square meter. The maximum power and work were derived from the respective power and work curves over the range of afterloads (5–50% P_o) examined.

After the completion of the force-velocity measurements, the muscle was stimulated repetitively under isotonic conditions (75 Hz in trains of 330-ms duration repeated each second) at 40% P_o , the load that produced maximum power in both CK $[-/-]$ and Ctl animals. Changes in velocity and extent of Dia shortening, power, and work were determined during repetitive isotonic activation. The endurance time (in s) was calculated as the interval between the initiation of repetitive isotonic activation and the cessation of muscle shortening. Following the repetitive isotonic activation paradigm, L_o was determined, and the stimulated muscle segment was weighed. Muscle cross-sectional area (CSA) was estimated on the basis of the following formula: muscle weight (g)/ $[L_o$ (cm) \times 1.056 (g/cm³)]. The estimated CSA was used to determine specific twitch (P_t/CSA) and tetanic (P_o/CSA) forces of the muscle segments.

Determination of CK activity and isoenzyme distribution. Total CK activity was determined at 25°C by using a coupled enzyme assay (34). The ATP generated by the CK reaction was used in a hexokinase/glucose-6-phosphate dehydrogenase-coupled enzyme system, which ultimately yields a reduced NADP (NADPH) proportional to CK activity (Sigma Diagnostics, St. Louis, MO). For this analysis, a 5- to 10-mg muscle sample was homogenized for 15 s in a 1:100 (wt/vol) dilution of CK extraction buffer containing 26 mM tris(hydroxymethyl)aminomethane (Tris), 0.3 M sucrose, 1% NP40, and 20 mM β -mercaptoethanol at pH 8.0. Homogenates were diluted to 1:100 in extraction buffer. Thirty-microliter aliquots of diluted homogenate were added to 1 ml of CK assay buffer at 25°C, containing 130 mM KCl, 10 mM Tris (pH 7.4), 1 mM MgCl₂, 2 mM AMP, 50 μ M diadenosine pentaphosphate, 5 mM glucose, 0.7 mM NADP, 1.5 mM ADP, 9 mM PCr, 1.3 U of hexokinase, and 0.5 U of glucose-6-phosphate dehydrogenase. AMP and diadenosine pentaphosphate were included to inhibit adenylate kinase from producing ATP. The rate increase in absorbance at 340 nm, due to production of NADPH through the coupled enzyme reaction, was used to determine CK activity. Care was taken to make sure that the rate obtained changed linearly with the volume of homogenate added. Protein content was determined by the Lowry method (15), and CK activity was expressed as micromoles per milligram protein per minute.

The CK isoenzyme phenotype was resolved electrophoretically (34). Homogenized muscle tissue (as above) was centrifuged for 20 min at 14,000 revolutions/min at 4°C, the supernatant was diluted 1:10 in extraction buffer, and 1 μ l of diluted supernatant was added to a 1% agarose gel (Ciba-Corning, Marshfield, MA). Electrophoresis was performed at 120 V for 20 min at 4°C. CK activity was visualized by evenly spreading Cardiotrac CK isoenzyme reagent (90 mM PCr, 60 mM magnesium acetate, 60 mM glucose, 60 mM *N*-acetyl cysteine, 15 mM AMP, 12 mM ADP, 6 mM NAD, 10 μ M diadenosine pentaphosphate, 9,000 U/l hexokinase, and 7,500 U/l glucose-6-phosphate dehydrogenase) on the gel and incubating for 20 min at 37°C. Production of NADPH in the gel was visualized directly with ultraviolet light. The BB, MB, MM, and mitochondrial CK isoforms are readily separated by this electrophoretic technique (34). To determine the relative contributions of individual isoforms to their respective total CK complements, photographs of the gels were taken and analyzed by using a scanning densitometer (GS 300 Hoefer

Scientific) and densitometry software (GS 365, Hoefer Scientific) to quantify the area under individual isoform peaks.

Determination of MHC composition. Myosin for electrophoresis was prepared by scissor-mincing the muscle tissue in a high salt solution, pH 6.5 at 4°C for 40 min (14). Extracts were centrifuged and supernatants recovered and treated as follows. Electrophoresis of MHC isoforms was performed by using the method of Talmadge and Roy (29). Ten microliters of supernatant were diluted (1:10) in a low-salt buffer consisting of 1 mM EDTA and 0.1% 2-mercaptoethanol (vol/vol) and stored overnight at 4°C to allow precipitation of myosin filaments. The filament solution was subsequently centrifuged to form a pellet, which was then dissolved in myosin sample buffer (0.5 M NaCl, 10 mM NaH₂PO₄) followed by dilution 1:100 in sodium dodecyl sulfate (SDS) sample buffer [62.5 mM Tris·HCl, 2% (wt/vol) SDS, 10% glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue at pH 6.8]. The samples were boiled for 2 min and stored at -80°C.

Gels were prepared from a stock solution of 30% acrylamide containing 29.4% (wt/vol) acrylamide and 0.60% (wt/vol) *N,N'*-methylene-bis-acrylamide (Bis). Electrophoresis was performed on slabs (18 × 16 cm × 0.75 mm thick) consisting of an 11.5-cm separating gel and a 4.5-cm stacking gel. Separating gels of total concentration of monomer (acrylamide + Bis) (T) = 8% and stacking gels of T = 4% at percentage of total monomer due to Bis (C) = 2% were used. Volumes of myosin extract (1–3 μl) containing 500–1,000 ng of protein/well were loaded on the gels. Electrophoresis (275 V for 3.5 h then 178 V for 17.5 h) was performed by using a vertical-slab gel unit (SE600, Hoefer Scientific Instruments) with Tris/glycine running buffer (29) in a cold room maintained at 4°C. Separating gels were silver stained (14). Monoclonal antibodies were used to identify the specific MHC bands (MHC_{slow}, MHC_{IIa}, MHC_{IIx}, MHC_{IIb}) separated on SDS-polyacrylamide gel electrophoresis gels and included 1) antibody BF-32 to MHC_{slow} and MHC_{IIa}; 2) antibody SC-71 to MHC_{IIa}; 3) BF-35 to all but MHC_{IIx}; 4) BF-F3 to MHC_{IIb}; and 5) RT-D9 to MHC_{IIx} and MHC_{IIb} (14). MHC gels were analyzed by using a scanning densitometer (GS 300 Hoefer Scientific) and densitometry software (GS 365, Hoefer Scientific) to quantify the area under individual isoform peaks. These data were used to determine the relative contributions of individual isoforms to their respective total heavy chain myosin complements.

Statistical analysis. Differences between groups with respect to animal weight and Dia muscle characteristics were determined by the unpaired Student's *t*-test. Statistical methods also included a two-way analysis of variance to compare changes in the velocity and extent of Dia shortening, power, and work as a function of study group and time during repetitive isotonic activations (Minitab Data Analysis Software, Minitab Windows version 9.2, State College, PA) (21). In the event of a significant analysis of variance, the Duncan multiple-range test was used as the post hoc analysis to define differences between control and CK[–/–] groups at specific times (36). Statistical significance was established a priori at *P* < 0.05. Data are reported as means ± SE.

RESULTS

Physical characteristics and isometric contractile properties. Animal and Dia characteristics, as well as Dia isometric contractile properties are presented in Table 1. Animal body weight, Dia *L*₀, CT, RT_{1/2}, P_t/CSA, and P_t/P₀ were not significantly different between CK[–/–] and Ctl animals (Table 1). Dia P₀/CSA, how-

Table 1. *Physical characteristics, isometric contractile properties, and force-velocity characteristics of Ctl and CK[–/–] double-deficient diaphragm*

	Ctl	CK[–/–]
Animal weight, g	27.7 ± 1.2	27.9 ± 1.0
<i>L</i> ₀ , mm	7.7 ± 0.1	7.7 ± 0.1
CT, ms	22.7 ± 0.7	22.6 ± 0.8
RT _{1/2} , ms	19.1 ± 1.1	19.7 ± 0.9
P _t /CSA, N/cm ²	5.5 ± 0.5	5.3 ± 0.4
P _t /P ₀	0.23 ± 0.03	0.26 ± 0.03
P ₀ /CSA, N/cm ²	21.9 ± 0.4	20.2 ± 0.4*
<i>V</i> _{max} , L ₀ /s	17.5 ± 1.1	14.1 ± 0.9*
<i>a</i> /P ₀	0.21 ± 0.02	0.21 ± 0.03
Power, W/m ²	2,744 ± 146	2,293 ± 138*
Work, J/m ²	284 ± 26	201 ± 9*

Values are means ± SE. Ctl, control; CK[–/–], creatine kinase-deficient diaphragm. *L*₀, optimal muscle length; CT, twitch contraction time; RT_{1/2}, twitch half relaxation time; P_t/CSA, twitch force normalized to muscle cross-sectional area; P_t/P₀, twitch force-to-tetanic force ratio; P₀/CSA, tetanic force normalized to muscle cross-sectional area; *V*_{max}, maximum velocity of shortening. **P* < 0.05 vs. Ctl diaphragm.

ever, was slightly lower (~8%) in CK[–/–] mice as compared with Ctl (Table 1).

Isotonic contractile properties. Dia force-velocity characteristics are also shown in Table 1. Mean force-velocity data from Ctl and CK[–/–] groups are shown in Fig. 1. Both groups manifested a hyperbolic force-velocity relationship (Fig. 1) with similar *a*/P₀ values (Table 1). *V*_{max}, however, was significantly lower (~19%) in CK[–/–] Dia compared with Ctl Dia (Table 1, Fig. 1).

Mean power-load data from control and CK[–/–] groups are shown in Fig. 2. Power manifested a parabolic pattern as a function of load, and maximum power was generated at 0.4–0.5 P₀ in both groups. Absolute maximum power, however, was lower in CK[–/–] mice, as compared with Ctl Dia (Table 1, Fig. 2), reflecting the greater specific force generated by Ctl Dia at all loads. Velocity of shortening at 40–50% P₀ was not different between groups.

Mean workload data from control and CK[–/–] Dia are shown in Fig. 3. Similar to the power-load relationship, work manifested a parabolic pattern as a function

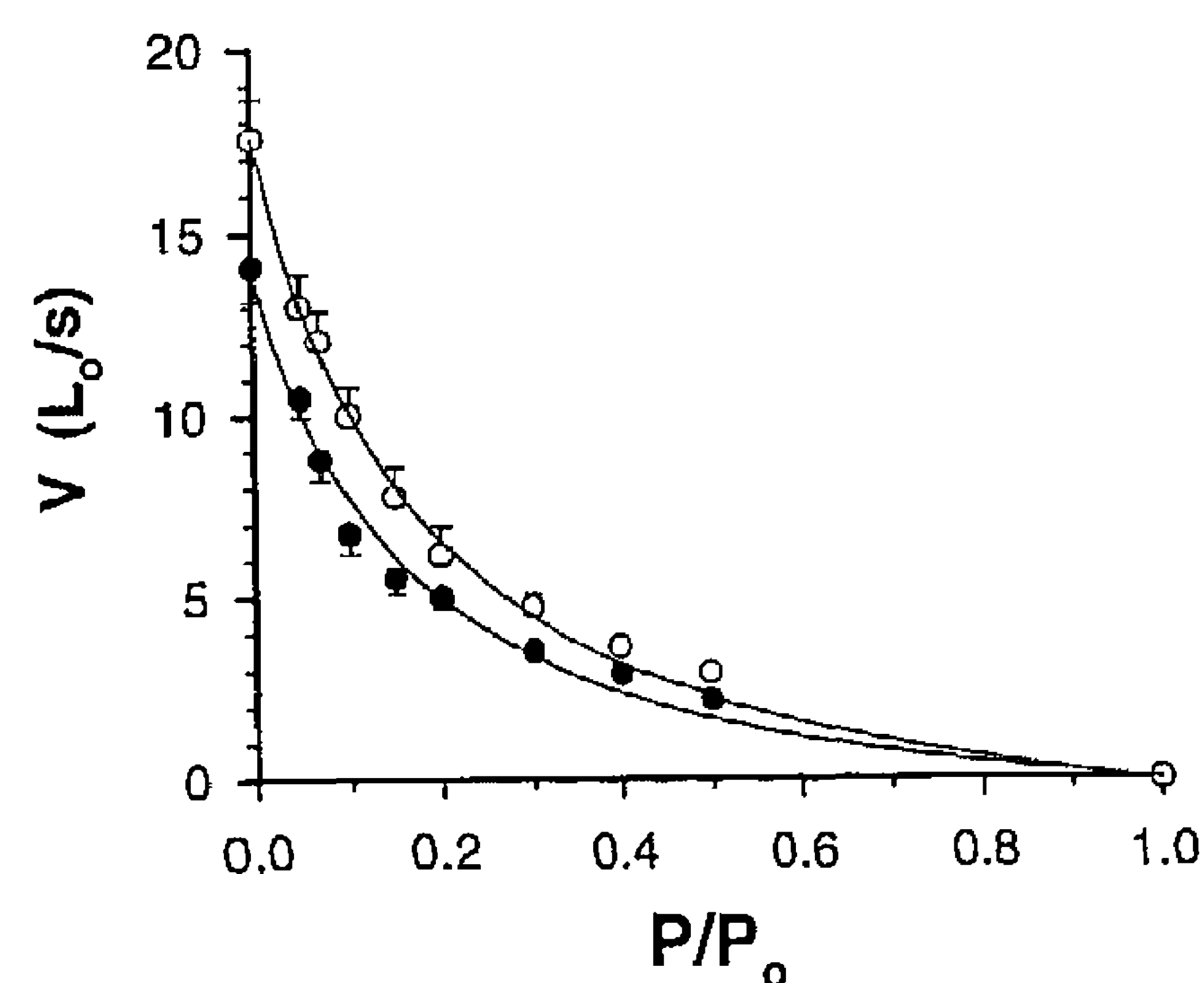


Fig. 1. Force (*P*/*P*₀, where *P*₀ is maximum tetanic force) -velocity (*V*) relationship of control (Ctl; ○) and creatine kinase-deficient (CK[–/–]; ●) diaphragm (Dia). Both groups manifested a hyperbolic force-velocity relationship with similar *a*/*P*₀ values (see Table 1). Maximum velocity of shortening was significantly lower in CK[–/–] Dia compared with Ctl Dia. *L*₀, optimal fiber length.

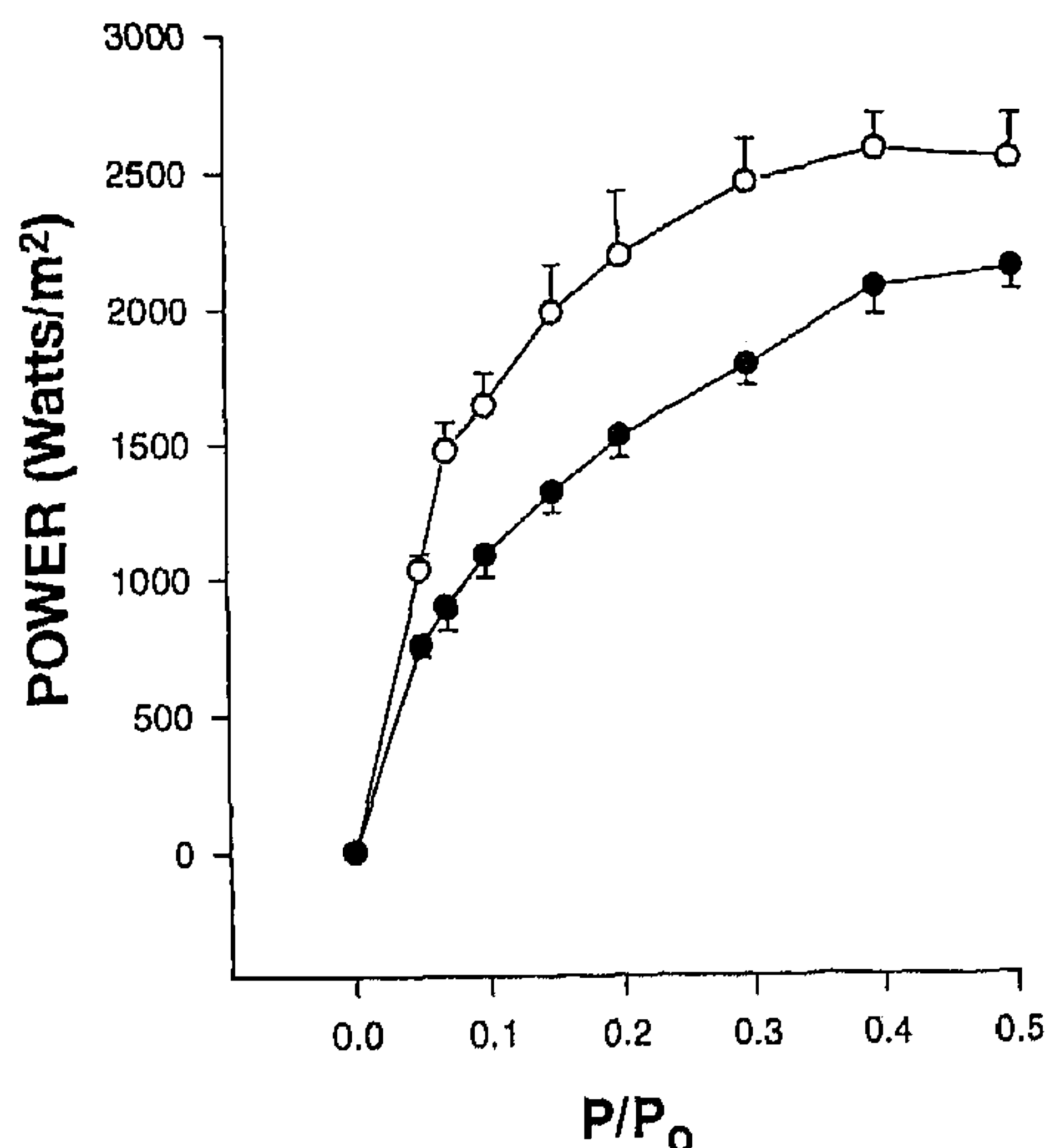


Fig. 2. P/P_0 -power relationship of Ctl (○) and CK[-/-] (●) Dia. Power manifested a parabolic pattern as a function of load. Absolute maximum power was significantly lower in CK[-/-] Dia compared with Ctl Dia.

of load, and maximum work was generated at 0.4–0.5 P_0 in both groups. Maximum work, however, was lower in CK[-/-] Dia, as compared with Ctl Dia, reflecting the greater P_0 /CSA generated by the Ctl Dia at all loads. The extent of Dia shortening at 40–50% P_0 was not different between groups.

The velocity and extent of muscle shortening, power, and work declined significantly during repetitive isotonic activation in both Ctl and CK[-/-] Dia (Fig. 4). The decline in power and work associated with repetitive isotonic activation reflected significant decreases in the velocity and extent of Dia shortening, respectively, as force was clamped at 0.4 P_0 during repetitive activation. The velocity and extent of shortening, power, and work after the initial stimulus were significantly lower in CK[-/-] as opposed to Ctl Dia (Fig. 4). These differences were sustained throughout the period of repetitive isotonic activation. Moreover, the endurance

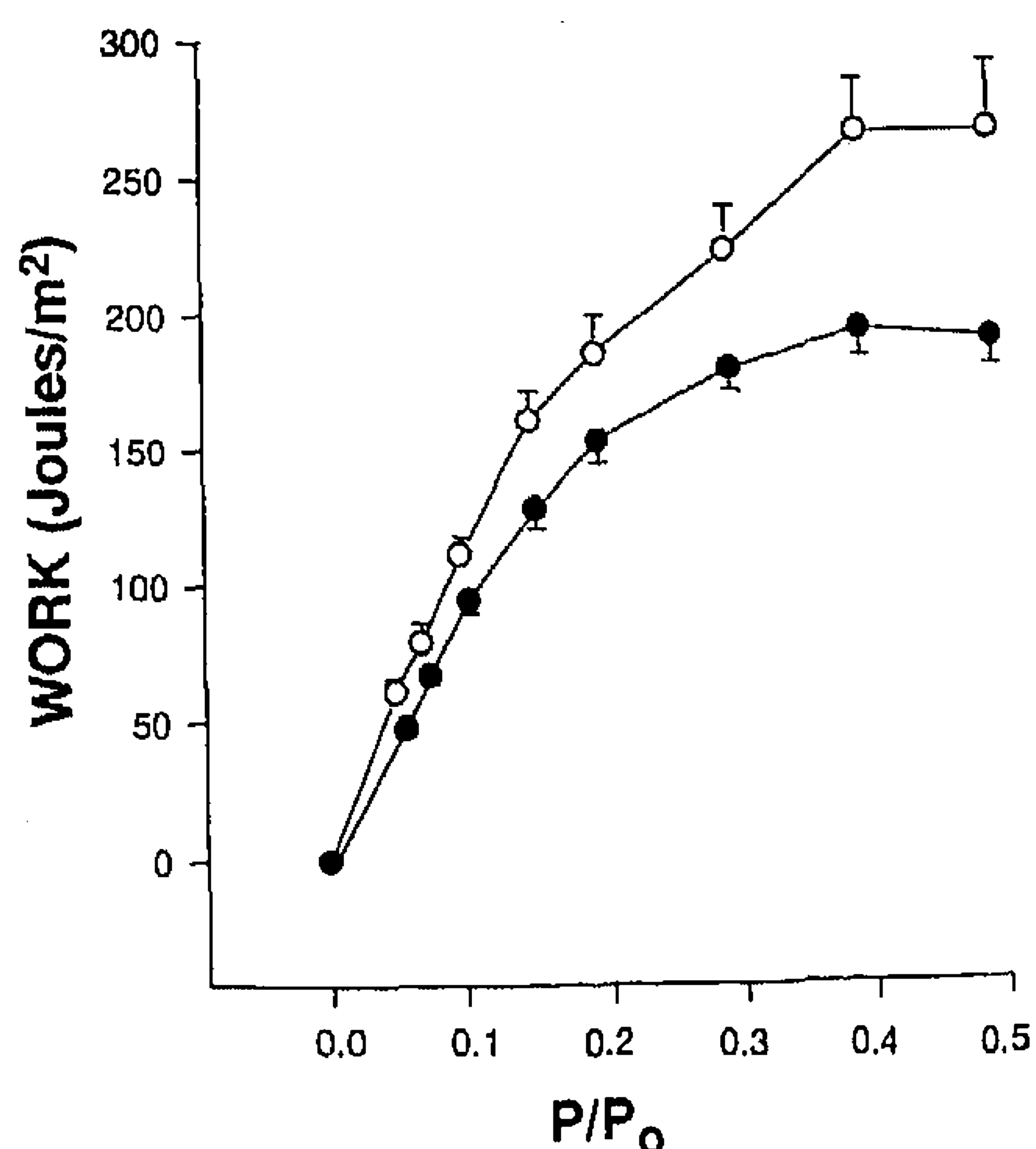


Fig. 3. P/P_0 -work relationship of Ctl (○) and CK[-/-] (●) Dia. Work manifested a parabolic pattern as a function of load. Absolute maximum work was significantly lower in CK[-/-] Dia compared with Ctl Dia.

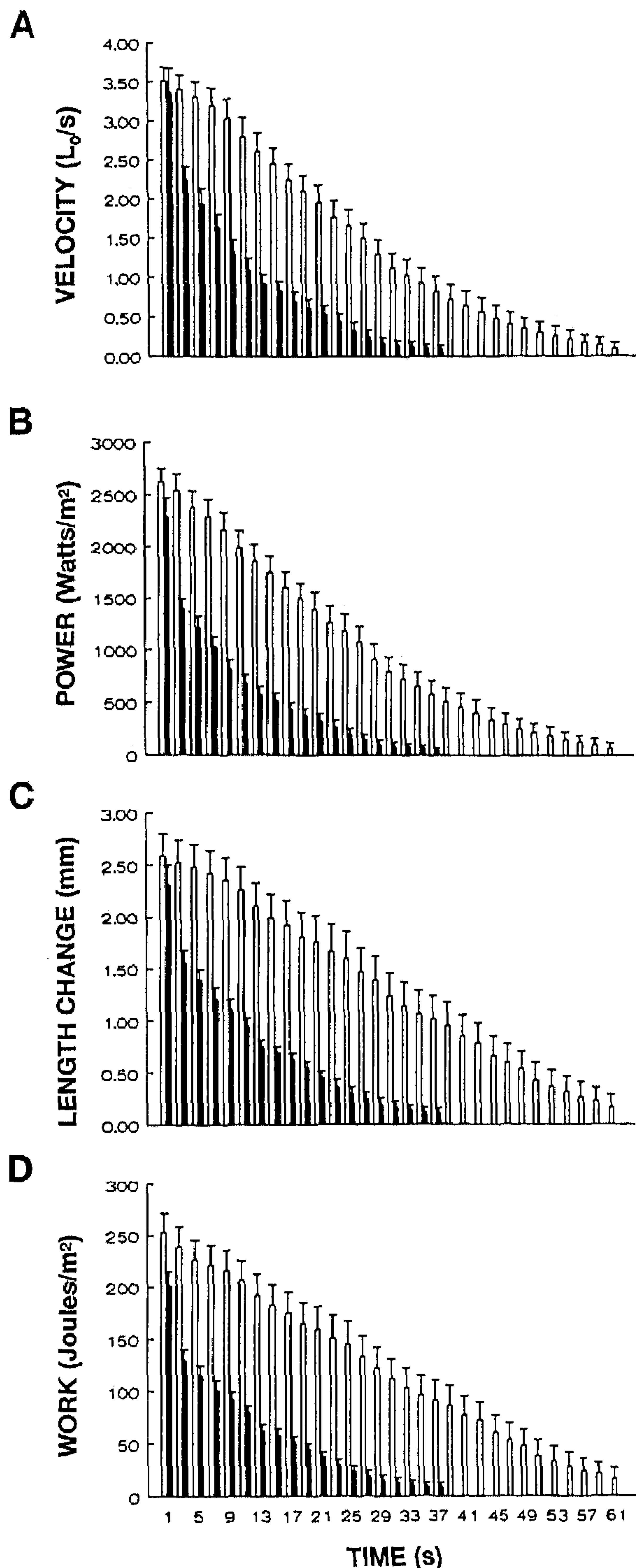


Fig. 4. Changes in velocity of shortening, power, extent of muscle shortening, and work of Ctl (open bars) and CK[-/-] (solid bars) Dia during repetitive isotonic activation. First stimulation and every other one thereafter are shown until muscle failed to shorten. Significantly lower velocities of shortening, power, length changes, and work were seen in CK[-/-] as opposed to Ctl Dia after initial stimulus. Interval between initiation of repetitive isotonic activation and cessation of muscle shortening, i.e., endurance time, was significantly shorter in CK[-/-] as opposed to Ctl Dia.

time of CK[-/-] Dia (37 ± 4 s) was significantly less than that of control Dia (61 ± 5 s) (Fig. 4).

Total CK activity and isoenzyme phenotype. Total CK activity was $801 \pm 42 \mu\text{mol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ in Ctl

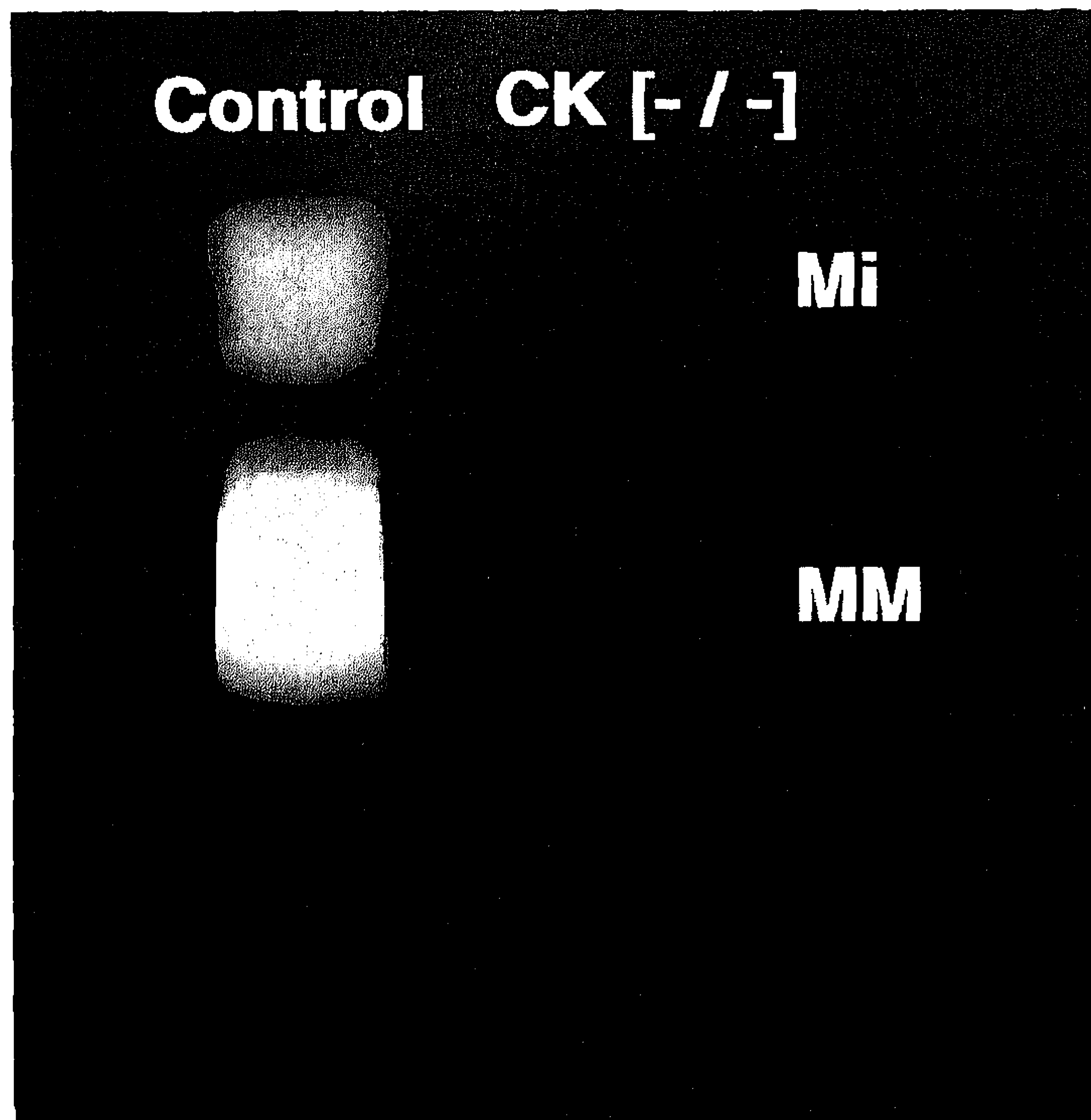


Fig. 5. Representative agarose gel stained for CK activity that demonstrates CK isoenzyme distribution [sarcomeric mitochondrial (ScCKmit; Mi), myofibrillar CK (M-CK; MM)] of Ctl and CK[-/-] Dia. Neither M-CK nor ScCKmit isoforms were noted in CK[-/-] Dia, as opposed to abundant expression of both isoforms in Ctl Dia.

Dia and was composed of ~25% ScCKmit and 75% CK-MM isoforms. In contrast, total CK activity in CK[-/-] Dia was $6.4 \pm 0.3 \mu\text{mol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$, averaging 0.8% of Ctl levels. Neither CK-M nor ScCKmit isoforms were noted on CK zymogen gels of CK[-/-] Dia, as opposed to abundant expression of both isoforms in Ctl Dia (Fig. 5), and we surmise residual CK activity to originate from immature satellite cells and the smooth muscle lining of vasculature in this muscle.

MHC phenotype. The MHC phenotypes of Ctl and CK[-/-] Dia were comparable and characterized by abundant MHC_{2A} and MHC_{2X} expression (Fig. 6). CK[-/-] Dia, however, expressed significantly less MHC_{2B} and more MHC_{slow} than its wild-type counterpart (Fig. 6).

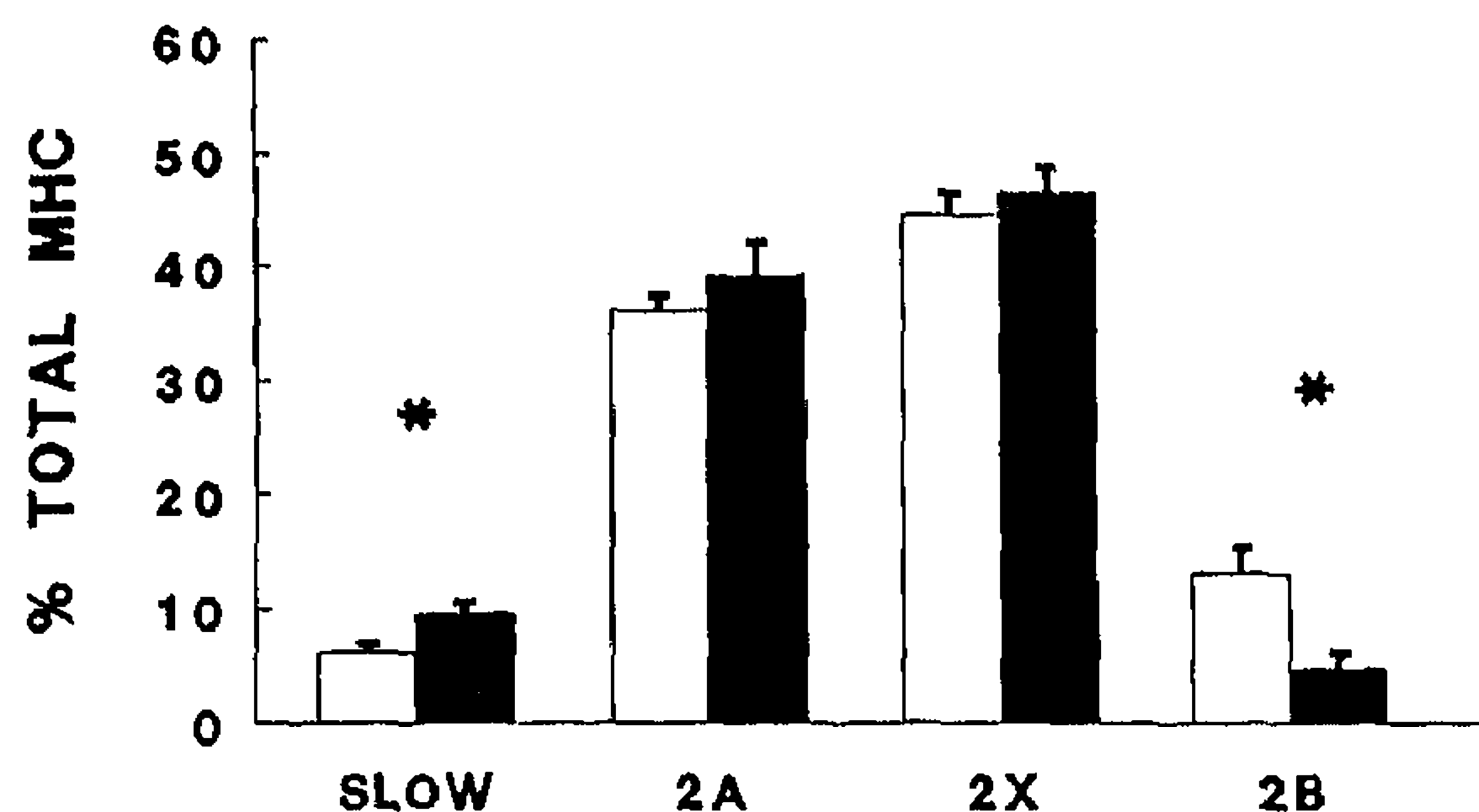


Fig. 6. Bar graph documents a densitometric comparison of myosin heavy chain (MHC) isoform expression in Ctl (open bars) and CK[-/-] (solid bars) Dia. Specific MHC isoform content is expressed as a relative %total MHC complement. * $P = 0.05$, Ctl vs. CK[-/-].

DISCUSSION

In vivo, the Dia shortens against a load, enlarging the chest cavity and generating negative intrathoracic pressure and air movement into the lungs. Thus two important functional properties of the Dia are power and work. The present study demonstrates that combined myofibrillar and sarcomeric mitochondrial CK deficiency is associated with a profound impairment of Dia power output and work performance during repetitive isotonic activation. These findings are consistent with the lower V_{max} and slightly reduced P_0/CSA noted in CK[-/-] Dia and underscore the importance of CK in respiratory muscle function.

Prior studies designed to assess the physiological role of CK in muscle have utilized nonspecific inhibitors of total CK activity such as dinitrofluorobenzene and iodoacetamide and suggest an important role for CK in muscle energy metabolism (11). Substrate analogs of Cr, e.g., β -guanidinopropionic acid (β -GPA), a poor substrate (higher Michaelis constant and lower V_{max}) for CK, have also been used to decrease PCr and ATP levels. Early studies on β -GPA-fed animals led to disparate results, with some investigations finding no effect of CK inhibition on skeletal muscle function (24) and others reporting an impairment of muscle force generation during repetitive activation (10, 16). More recent studies on β -GPA-fed animals demonstrate a greater impairment of limb muscle isometric force-generating capacity during the initial series of repetitive activation, as compared with control muscle (17). Force generation during the latter phases of repetitive activation, however, was stabilized at control levels or even higher in β -GPA-fed animals (17). Similar observations have been made in M-CK-deficient transgenic mouse limb muscle (30, 31). In β -GPA-fed animals, the loss of force-generating capacity during the early phase of activation likely reflects their diminished skeletal PCr and ATP stores, whereas their ability to sustain force during the latter phases of repetitive activation mirrors skeletal muscle structural and biochemical adaptations to chronic β -GPA exposure. Such adaptations include 1) type II fiber atrophy (23); 2) an increase in MHC_{slow} isoform expression (19); 3) an increase in mitochondrial enzyme activity (23), and 4) a decrease in glycolytic potential (23). Analogous observations have been reported in M-CK-deficient transgenic mouse limb muscle (27, 30, 31). The aforementioned changes suggest that the skeletal muscle of β -GPA-fed animals is able to adapt, so as to activate pathways of aerobic metabolism in response to a diminished creatine pool and thereby preserve function, and have been interpreted as evidence of the importance of CK in skeletal muscle high-energy metabolism.

In the present study, we used an alternative approach to examine the role of CK in respiratory muscle function, i.e., ScCKmit/M-CK double-deficient mutant mice generated via targeted mutagenesis (27). More specifically, mice bearing a null mutation of the ScCKmit gene (27, 28) were interbred with mice bearing a null mutation of the M-CK gene (32) to generate

heterozygotes for both CK isoforms. Sibling matings of these mice resulted in the generation of a CK[−/−] double-deficient line (27).

Previous studies have explored the contractile and fatigue characteristics of the medial gastrocnemius in wild-type, M-CK-deficient (7, 27, 30, 31), ScCKmit-deficient (27, 28), and CK[−/−] double-deficient (27) transgenic mice. These studies indicated no differences in P_t , CT, or $RT_{1/2}$ of the twitch contractions among the four CK phenotypes (27). However, these studies suggested that CK might modulate the contractile response of the medial gastrocnemius during repetitive isometric (7, 27, 30, 31) or isovelocity (7) activations. Medial gastrocnemius from M-CK-deficient and CK[−/−] double-deficient mice failed to sustain twitch and tetanic force production during the early phase of repetitive activation, while forces stabilized at the same level as controls during the latter stages of repetitive contraction. The absence of ScCKmit alone was not associated with any significant change in force generation during any stage of repetitive isometric activation as compared with control muscle (27, 28), suggesting that the absence of M-CK accounted for the impairment in function of CK[−/−] medial gastrocnemius. Indeed, a recent study of transgenic mouse mutants with graded reductions in M-CK activity showed a strong positive correlation between the level of M-CK expression in limb muscle and the ability to maintain maximal muscle force during the initial phase of isometric activation (31).

In contrast, in preliminary studies, we were unable to demonstrate that M-CK deficiency alone was associated with any greater impairment in Dia force-generating capacity than control Dia during repetitive isometric twitch or tetanic stimulation (13). This discrepancy may relate to the relatively abundant expression of ScCKmit in the Dia [$\sim 25\%$ of the total CK phenotype (34)], as opposed to medial gastrocnemius muscle (1–2% of the total CK phenotype). The enhanced expression of ScCKmit in the Dia may provide sufficient ATP-buffering capacity to counter the effects of isolated myofibrillar CK deficiency in the M-CK-deficient transgenic Dia. We, therefore, sought to determine the effect of combined M-CK and ScCKmit deficiency on Dia function and to do so within the context of repetitive dynamic activation where metabolic demands would be highest (9). Indeed, the repetitive isotonic activation protocol used in the present study creates a condition that emphasizes the role of CK in meeting the energy demands of contraction, although it may not strictly mimic the normal physiological situation.

Isometric twitch characteristics were not different between CK[−/−] and Ctl Dia. In contrast, P_o/CSA was significantly lower in CK[−/−] Dia as compared with Ctl Dia. The mechanism underlying the slight reduction of P_o/CSA in CK[−/−] Dia was not addressed by the present study, but this finding is consistent with the aforementioned observations on CK[−/−] limb muscle (medial gastrocnemius) (27) and β -GPA-treated rats (16).

The present study was the first to examine the effect of CK deficiency on the force-velocity relationship of the Dia. We observed a significantly lower V_{max} in CK[−/−] as opposed to Ctl Dia. The curvature of the force-velocity relationship as quantified by a/P_o , however, was not different between study groups. V_{max} is highly correlated with the rate of ATP hydrolysis (actomyosin ATPase activity) by the MHC (4) and reflects the maximal cross-bridge cycling rate. The mechanism underlying the lower V_{max} in CK[−/−] Dia may relate in part to the alteration in MHC phenotype of the Dia in response to combined CK-M and ScCKmit deficiency, i.e., an increased MHC_{slow} and diminished MHC_{2B} expression. This shift in MHC isoform expression, also observed in β -GPA-treated animals (19), might be expected to decrease the overall actomyosin ATPase activity of the CK[−/−] Dia, as well as cross-bridge cycling rate and V_{max} . However, the difference in MHC isoform expression between Ctl and CK[−/−] Dia was relatively small and, therefore, unlikely to fully account for the difference in V_{max} . Other possible contributing factors to the difference in V_{max} between Ctl and CK[−/−] Dia include alterations in regulatory contractile protein isoforms, i.e., alkaline myosin light chain composition (5) and troponin. Additional investigation is necessary to clarify this issue.

The CK[−/−] Dia was less able to sustain power (shortening velocity) and work (extent of shortening) during repetitive isotonic activation, compared with Ctl Dia. Shortening velocity and the extent of shortening were already significantly depressed in the second series of isotonic activation, and this depression was persistent throughout the period of repetitive stimulation. The rapidity of this decline was marked and consistent with previous reports on CK[−/−] limb muscle during repetitive activation (27). Moreover, the interval between the initiation of repetitive isotonic activation and the cessation of muscle shortening, i.e., endurance time, was significantly shorter in CK[−/−] as opposed to Ctl Dia. The initial cross-bridge cycling rates did not differ between Ctl and CK[−/−] Dia at the afterload used during the repetitive isotonic activation paradigm, indicating that the rate of ATP consumption should be similar between groups at the start of the endurance protocol. The subsequent rapid decline in power and work output of CK[−/−] as opposed to control Dia during repetitive isotonic activation likely reflects the high energetic demands of dynamic contractions and the impaired ATP-buffering capacity of CK[−/−] Dia. The latter may limit actomyosin ATPase activity with a resultant decrease in cross-bridge cycling rate, velocity of shortening, and power in CK[−/−] Dia.

The decreased velocity of shortening may also relate to intracellular metabolic changes during repetitive isotonic activation that could be accentuated in CK[−/−] Dia. More specifically, the accumulation of metabolites during repetitive muscle activation such as MgADP and hydrogen ions have been shown to impair the kinetic properties of skinned muscle fibers (1, 6, 8), and the concentration of these products should be

enhanced in the absence of CK ATP-buffering capacity. On the basis of measurements of the V_{\max} of diaphragm actomyosin ATPase activity (26), $\sim 0.8 \mu\text{mol}$ of ATP/g tissue should be hydrolyzed during 330 ms of isotonic activation. Without adequate CK buffering of ADP, this would cause a large increase in ADP concentration after even one contraction. Analogous calculations by others suggest that, in the absence of CK buffering, ADP concentrations rise during muscle activation to levels that significantly reduce the shortening velocity of skinned fibers in vitro (1, 6). Finally, although there is only limited evidence to indicate that myoplasmic free calcium concentration influences shortening velocity (1), we have recently observed that CK[−/−] Dia has distinctly altered calcium release and sequestration characteristics (unpublished observations) during repetitive activation that may have profound effects on the excitation-contraction profile of this muscle. Additional investigation is necessary to clarify which factor or combination of factors is primarily responsible for the marked decline in power output and work performance in CK[−/−] Dia during repetitive isotonic activation.

In summary, the results of the present study demonstrate that combined ScCKmit/M-CK deficiency is associated with 1) a slight decrease in P_0/CSA , 2) a decrease in V_{\max} , 3) a decrease in maximum power and work, and 4) a profound impairment of Dia shortening capacity during repetitive isotonic activation, as compared with wild-type Ctl. Both the extent and velocity of Dia shortening during repetitive isotonic activation in CK[−/−] Dia were markedly diminished, leading to significantly greater declines in power output and work performance over time as compared with Ctl. These findings underscore the functional importance of CK in skeletal muscle.

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