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RESEARCH ARTICLE

Myosin phosphorylation improves contractile economy of mouse fast skeletal muscle during staircase potentiation

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ABSTRACT

Phosphorylation of the myosin regulatory light chain (RLC) by skeletal myosin light chain kinase (skMLCK) potentiates rodent fast twitch muscle but is an ATP-requiring process. Our objective was to investigate the effect of skMLCK-catalyzed RLC phosphorylation on the energetic cost of contraction and the contractile economy (ratio of mechanical output to metabolic input) of mouse fast twitch muscle *in vitro* (25°C). To this end, extensor digitorum longus (EDL) muscles from wild-type (WT) and from skMLCK-devoid (skMLCK$^{-/-}$) mice were subjected to repetitive low-frequency stimulation (10 Hz for 15 s) to produce staircase potentiation of isometric twitch force, after which muscles were quick frozen for determination of high-energy phosphate consumption (HEPC). During stimulation, WT muscles displayed significant potentiation of isometric twitch force while skMLCK$^{-/-}$ muscles did not (i.e. 23% versus 5% change, respectively). Consistent with this, RLC phosphorylation was increased ∼3.5-fold from the unstimulated control value in WT but not in skMLCK$^{-/-}$ muscles. Despite these differences, the HEPC of WT muscles was not greater than that of skMLCK$^{-/-}$ muscles. As a result of the increased contractile output relative to HEPC, the calculated contractile economy of WT muscles was greater than that of skMLCK$^{-/-}$ muscles. Thus, our results suggest that skMLCK-catalyzed phosphorylation of the myosin RLC increases the contractile economy of WT mouse EDL muscle compared with skMLCK$^{-/-}$ muscles without RLC phosphorylation.

KEY WORDS: Regulatory light chain, Myosin light chain kinase, Isometric twitch, Energetics

INTRODUCTION

The striated muscle cells that form skeletal muscle are biological motors that convert chemical energy into mechanical forces to power locomotion such as running, swimming and flight. Within each sarcomere, calcium (Ca^{2+})-regulated interactions between myriad myosin motor molecules and their designated docking sites on sarcomeric myofilaments transduce the energy provided by the hydrolysis of adenosine triphosphate (ATP) into force and work. The ability of cycling cross-bridges to generate the inter-filament forces required for muscle shortening and thus locomotion depends upon the ability of intracellular Ca^{2+} signals to activate cross-bridge formation in a feed-forward manner, while the availability of ATP and/or inhibitory effect of ATP hydrolysis product accumulation may limit cross-bridge formation cycling via metabolic feed-back (Lymn and Taylor, 1971).

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Received 4 August 2017; Accepted 31 December 2017

The intracellular Ca^{2+} signal that regulates cross-bridge formation also activates skeletal myosin light chain kinase (skMLCK), a phosphotransferase enzyme responsible for introducing a negative charge to a serine residue on the myosin regulatory light chain (RLC) unit (Rayment et al., 1993; Sweeney et al., 1994). In turn, skMLCK-catalyzed phosphorylation of the RLC may induce structure–function changes to myosin that modulate the ability of Ca^{2+} to regulate force (see Vandenboom, 2016). As an example, skMLCK-catalyzed phosphorylation of the RLC increases the Ca^{2+} sensitivity of force in permeabilized skeletal muscle fibers (Metzger et al., 1989; Persechini et al., 1985; Sweeney and Kushmerick, 1985). Importantly, although steady-state force is enhanced, the force:ATPase relationship is not (Sweeney and Stull, 1990), suggesting that phosphorylation of the RLC promotes the ability of cycling cross-bridges to attain a force-generating state independent of any change to myosin ATPase rate (e.g. Morgan et al., 1976).

Myosin phosphorylation-mediated alterations to cross-bridge structure that increase the Ca^{2+} sensitivity of the contractile apparatus may also modulate force output of intact skeletal muscle, either *in situ* or *in vitro* (reviewed by MacIntosh, 2003). For example, strong associations between RLC phosphorylation and contraction-induced increases in muscle force and/or work, i.e. muscle potentiation, have been presented in a number of rodent skeletal muscle models (reviewed by Vandenboom et al., 2013). Moreover, fast twitch muscles from skMLCK knockout mice that do not display contraction-induced elevations in RLC phosphorylation display greatly reduced levels of potentiation compared with their wild-type (WT) counterparts (Bowslaugh et al., 2016; Gittings et al., 2011, 2017; Zhi et al., 2005). Although redundant mechanisms for potentiation may exist, an important question is whether skMLCK-catalyzed phosphorylation of the RLC significantly alters the contractile economy (i.e. mechanical output:metabolic input) of potentiated skeletal muscle. Crow and Kushmerick (1982) were the first to examine this relationship, and reported a positive correlation between RLC phosphorylation and reduced energy cost for isometric tetani of mouse extensor digitorum longus (EDL) muscle (*in vitro*, 22°C). However, subsequent work on rat gastrocnemius muscle (*in situ*, 35°C) showed that RLC phosphorylation had no consistent relationship with energy consumption during isometric tetani (Barsotti and Butler, 1984) or cross-bridge cycling rate during unloaded shortening (Butler et al., 1983). More recently, Abbate et al. (2001) found that potentiation had a greater effect on high-energy phosphate turnover than on work, thus reducing the economy of rat gastrocnemius muscle (*in situ*, 35°C). An important point was that it was the extra work caused by potentiation, and not the energetic cost of RLC phosphorylation per se, that decreased economy. Thus, no concordance in the literature exists regarding the influence of RLC phosphorylation-mediated potentiation on the contractile economy of rodent fast twitch muscle.

A limitation of previous studies examining the interaction of myosin RLC phosphorylation-mediated potentiation and contractile

Journal of Experimental Biology (2018) 221, jeb167718. doi:10.1242/jeb.167718

List of abbreviations

BTS	*N*-benzyl-*p*-toluene sulfonamide
Cr	creatine
$-dP/dt$	rate of force relaxation
$+dP/dt$	rate of force development
E_A	activation energy
E_{total}	total energy turnover
E_{XB}	cross-bridge energy turnover
EDL	extensor digitorum longus
FTI	force–time integral
HEPC	high-energy phosphate consumption
L_o	optimal length (for peak muscle twitch force)
PCr	phosphocreatine
P_t	peak force
RLC	regulatory light chain
$RT_{1/2}$	half-relaxation time
skMLCK$^{-/-}$	myosin light chain kinase knockout (skeletal muscle isoform)
TPT	time to peak tension
V_{max}	maximal shortening velocity

economy of rodent fast twitch muscle is the lack of a robust negative control for skMLCK activity and RLC phosphorylation. This is because even brief stimulation of most rodent fast twitch skeletal muscles results in fractional activation of skMLCK, making it difficult to parse out the influence of RLC phosphorylation from other processes. The purpose of this study was to compare the contractile economy of WT muscles and skMLCK$^{-/-}$ muscles lacking the enzymatic ability to phosphorylate myosin RLC. To do this, we used a paradigm that replicated the onset of low-frequency activity, a situation where the energetic cost of myosin phosphorylation, and its influence on economy, should be greatest. The use of different genotypes allowed us to parse out the influence of RLC phosphorylation on potentiation and high-energy phosphate consumption. We hypothesized that WT muscles with myosin phosphorylation would potentiate more than skMLCK$^{-/-}$ muscles, but that energy consumption would be increased in proportion to this increase in mechanical output, thus maintaining contractile economy compared with skMLCK$^{-/-}$ muscles without myosin phosphorylation. This may be a critical teleological question as a large decrease in economy may limit the usefulness of the potentiated state for working skeletal muscle (Brown and Loeb, 1998; Tsianos and Loeb, 2014).

MATERIALS AND METHODS

Study design

This study was approved by the Brock University Animal Care Committee and conforms to the standards of the Canadian Council for Animal Care. Adult skMLCK$^{-/-}$ mice (*Mus musculus* Linnaeus 1758, C57BL/6 background, 19.4±0.7 g, 8–12 weeks old, n=20) were obtained from our breeding colony at Brock University. WT C57BL/6 mice (*Mus musculus*, 19.4±0.3 g, 8–12 weeks old, n=20) were purchased from Charles River Laboratories (Saint Constant, QC, Canada). Identical experiments were conducted on the two genotypes to determine contractile economy with and without the ability to phosphorylate myosin RLC, and to investigate potential differences in total energy turnover (E_{total}), as well as energy turnover for contractile (cross-bridge energy turnover, E_{XB}) and non-contractile (activation energy, E_A: i.e. SERCA and Na^+/K^+-ATPase) processes. A major limitation of rapid freezing followed by chemical analysis of extracts of the frozen muscle is that each muscle provides only a single measurement, at a single time point. Because it was impossible to adopt a repeated measures design, half of the muscles were used for determination of myosin RLC phosphorylation and the other half were used for measurement of high-energy phosphate consumption (HEPC) (i.e. multiple independent measurements).

Contractile experiments and analysis

On the day of an experiment, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg kg^{-1} body mass). Bilateral EDL muscles were surgically isolated from each hindlimb of WT and skMLCK$^{-/-}$ mice and immediately (<5 s) suspended in a vertical jacketed organ bath (Radnoti LLC, Monrovia, CA, USA) containing continuously oxygenated Tyrode's solution (Lännergren et al., 2000) maintained at 25.0±0.1°C using an Isotemp 4100 R20 circulator (Fisher Scientific Company, Ottawa, ON, Canada). Contractile experiments were conducted using a 1200a In Vitro Muscle Testing System and field stimulation was applied using flanking platinum electrodes driven by a Model 701B biphase stimulator (Aurora Scientific, Inc., Aurora, ON, Canada) with voltage set to 1.25 times the threshold required to activate all fibers and elicit maximal isometric twitch force. Muscle length was measured using digital vernier calipers with LCD display. All contractile data were sampled and collected at 2000 Hz from a 305B servomotor acquired through a 604C analog-to-digital interface, and controlled by a dual-mode lever system (Aurora Scientific, Inc.). Data acquisition and basic analysis were performed using Aurora Scientific, Inc. 600a software (v1.60) and further examined using Excel (Microsoft Canada Co., Mississauga, ON, Canada). After mounting, muscles were allowed a 30 min equilibration period, after which optimal length (L_o) for maximal isometric twitch force was determined. The muscle length was adjusted to 0.9 L_o to maximize the magnitude of isometric twitch potentiation (Rassier and MacIntosh, 2000), and following an 80 min period of rest, the muscle was repetitively stimulated at 10 Hz for 15 s for a total of 150 individual twitches (Fig. 1). In a subset of muscles, stimulation was performed in the presence of BTS (*N*-benzyl-*p*-toluene sulfonamide), a myosin ATPase inhibitor that does not interfere with intracellular Ca^{2+} handling (Pinniger et al., 2005; Shaw et al., 2003). In either case, relative twitch force was quantified as the ratio of final twitch force to initial twitch force, producing a value that reflects an increase or decrease in twitch force relative to baseline measures. Each experiment was terminated when the EDL was rapidly frozen (<2 s) using tongs pre-cooled in liquid nitrogen. Muscles were then stored at −80°C for future determination of either RLC phosphorylation or metabolite concentrations (with or without BTS).

Analysis of twitch characteristics

A rapid increase in force occurred over the initial 0.5 s of repetitive stimulation due to partial fusion of twitches and thus final twitch force was normalized to the twitch occurring at 0.5 s (i.e. the 6th twitch in the series) (Ryder et al., 2007). The initial, 25th, 50th and final twitch of repetitive stimulation were analyzed for peak force (P_t), time to peak tension (TPT), half-relaxation time ($RT_{1/2}$), rate of force development ($+dP/dt$) and rate of force relaxation ($-dP/dt$). As all twitches were isometric (i.e. fixed length), we could not quantify work per se; instead, the force–time integral (FTI) was calculated and the area under the force–time curve was used as a surrogate for isometric work (de Haan et al., 1986).

Myosin RLC phosphorylation

In parallel experiments, a subset of muscles were removed from −80°C storage and myosin RLC phosphate content was quantified

Journal of Experimental Biology (2018) 221, jeb167718. doi:10.1242/jeb.167718

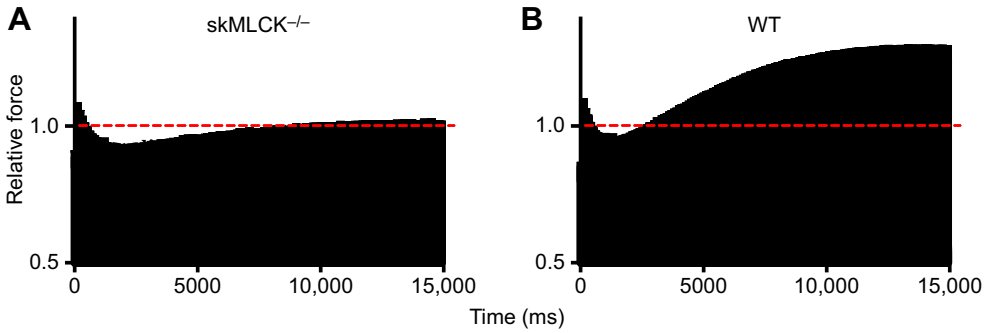

Fig. 1. Representative force traces during repetitive low-frequency stimulation (*in vitro*, 25°C). (A) Mice lacking skeletal muscle myosin light chain kinase (skMLCK$^{-/-}$). (B) Wild-type (WT) muscles. Muscles were stimulated at 10 Hz for 15 s to induce staircase potentiation at 0.90 optimal length (L_o). In these examples, the final twitch was 1.04 and 1.60 of baseline values in the skMLCK$^{-/-}$ and WT muscle, respectively. Note also that the depression in isometric twitch force (i.e. negative staircase) evident over the first several pulses is considerably reduced in amplitude and duration in the WT muscle compared with the skMLCK$^{-/-}$ muscle.

by urea–glycerol PAGE followed by immunoblotting. These methods were based on Zhi et al. (2005) and have been described in detail previously (see Gittings et al., 2016; Bowslaugh et al., 2016). The primary myosin RLC antibody used in this work was graciously donated by Dr Jim Stull and his lab at the University of Texas Southwestern Medical Center.

HEPC

Muscles were removed from −80°C storage and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) for a minimum of 6 h to remove all water. After the silk sutures were removed, the muscles were dissected free of connective tissue and blood, then powdered and aliquoted into 1.5 ml microcentrifuge tubes. Metabolites were extracted from aliquots of lyophilized tissue using 0.5 mol l^{-1} perchloric acid ($HClO_4$), and neutralized with 2.3 mol l^{-1} potassium carbonate ($KHCO_3$). The concentrations of metabolites were analyzed in triplicate in opaque black polypropylene 96-well microplates (Greiner Bio-One America Inc., Monroe, NC, USA) using fluorometric techniques as previously described (Bergmeyer et al., 1983; Harris et al., 1974). Three specific assays were used to identify the concentration of the metabolites of interest, including ATP, phosphocreatine (PCr), creatine (Cr) and lactate. To adjust for variability in solid non-muscle constituents, all raw metabolite values were normalized to total Cr content (Zhang et al., 2006). HEPC, a measure of ATP turnover determined through anaerobic ATP production, was calculated for each group from changes of metabolite concentration using Eqn 1:

$$\text{HEPC} = 1.5\Delta\text{Lactate} - \Delta - 2\Delta[\text{ATP}]. \quad (1)$$

The change (Δ) signifies the differences in metabolite concentration between the experimental muscle and its contralateral resting control. The calculation for HEPC thus uses an equation that assumes that glycogen is the sole source for lactate production during contraction and any decrease in ATP within the muscle is associated with a stoichiometric increase in inosine monophosphate (IMP) (Zhang et al., 2006). Energy balance experiments show that the contribution to overall ATP production by oxidative metabolism is no more than 9% during brief contractile activity (<30 s) (Westra et al., 1988) and thus this contribution to energy turnover was ignored. Although we used a 15 s stimulation protocol in order to increase PCr breakdown and reduce uncertainty in its measurement, we cannot exclude entirely the possibility that errors in its measurement are propagated through calculations of HEPC.

Calculation of contractile economy

Contractile economy of both genotypes was calculated as the ratio of total FTI obtained during low-frequency stimulation to HEPC, as measured by NADH-linked fluorometric assays. Parallel experiments were conducted in which BTS was used to pharmacologically inhibit myosin II ATPase activity (Shaw et al., 2003). This procedure allowed us to partition E_{total} into E_{XB} and E_A components after directly measured HEPC values were corrected for residual force production during the BTS incubation using Eqn 2 (Zhang et al., 2006):

$$E_{\text{XB,corrected}} = \left(\frac{E_{\text{total}} - E_{\text{A}}}{E_{\text{total}}}\right)\left(\frac{100\%}{\%\ \text{Force inhibition}}\right)100\%. \quad (2)$$

Once this was done, new corrected HEPC values for cross-bridge and E_A components were calculated. Contractile economy was defined as the ratio of mechanical output to metabolic input, i.e. FTI to HEPC, and expressed as millinewton-seconds per micromole of high-energy phosphate (mN s μmol^{-1} P) (de Haan et al., 1986). BTS was solubilized in dimethyl sulfoxide (DMSO) to create a 25 mmol l^{-1} stock solution. A small volume of BTS stock solution was added to the organ bath following preliminary procedures to yield a final concentration of 25 μmol l^{-1}. Muscles were incubated in BTS for 80 min before the contractile experiment began.

Statistics

After checking for normal distribution and homogeneity of variances, a two-way repeated measures ANOVA was conducted to evaluate the effect of skMLCK expression (i.e. WT or skMLCK$^{-/-}$) and repetitive stimulation (i.e. resting or stimulated) on isometric twitch force and work in the control condition. A two-way ANOVA was conducted to compare the effect of skMLCK expression and repetitive stimulation on RLC phosphorylation. Two-tailed, unpaired *t*-tests were conducted to examine the effect of skMLCK expression on total FTI, E_{total}, E_{XB} and E_A and contractile economy. All statistical analyses were conducted using GraphPad Prism 6 (La Jolla, CA, USA) with significance set at $P<0.05$. All data are means±s.e.m. unless otherwise stated. Sample sizes (n) used to achieve statistical power were based upon previous work from our lab and others for both mechanical (Gittings et al., 2011, 2012, 2016; Abbate et al., 2001) and biochemical (Zhi et al., 2005; Zhang et al., 2006) analyses.

Journal of Experimental Biology (2018) 221, jeb167718. doi:10.1242/jeb.167718

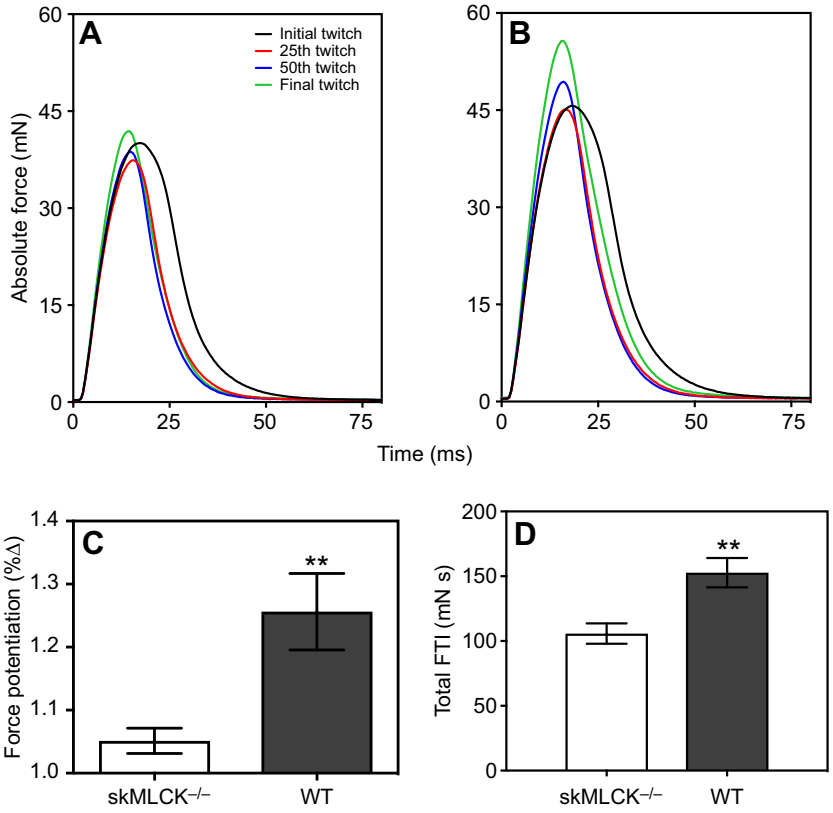

Fig. 2. Contractile data from WT and skMLCK$^{-/-}$ muscles subjected to repetitive low-frequency stimulation (*in vitro*, 25°C). (A,B) Representative isometric twitch force records of extensor digitorum longus (EDL) muscles from skMLCK$^{-/-}$ (A) and WT (B) mice at selected times during repetitive low-frequency stimulation. (C,D) Summary of relative change in mean isometric twitch force (C; means±s.e.m., n=8, **P<0.01 for one-tailed Mann–Whitney rank sum test), and force–time integral (FTI; D; means±s.e.m., n=8, **P<0.01 for one-tailed unpaired t-test) during repetitive stimulation of skMLCK$^{-/-}$ and WT muscles.

RESULTS

Effect of repetitive stimulation on isometric twitch force in WT and skMLCK$^{-/-}$ muscles

Representative twitch records obtained at select time points are shown in Fig. 2A,B and relative changes to WT and skMLCK$^{-/-}$ muscles during repetitive stimulation are summarized in Fig. 2C. Initial absolute isometric twitch force was similar between WT and skMLCK$^{-/-}$ muscles (45.8±3.5 and 40.2±2.8 mN, respectively, P=0.340); however, with repetitive stimulation, WT absolute isometric twitch force increased significantly more than skMLCK$^{-/-}$ values (56.3±2.4 and 42.2±3.0 mN, respectively, P<0.01). In addition to changes to peak force, the TPT and $RT_{1/2}$ of twitches of each genotype decreased from the first to the final twitch in the series (Table 1). When genotypes are compared, WT TPT was significantly higher at each time point compared with that of skMLCK$^{-/-}$ muscles. By contrast, the $RT_{1/2}$ of WT muscles was greater than that of skMLCK$^{-/-}$ muscles at the first and final twitches only. With respect to twitch kinetics, the initial +dP/dt and −dP/dt of each genotype was similar and each increased with repetitive stimulation; however, +dP/dt was increased more in the WT than in the skMLCK$^{-/-}$ genotype while the change to −dP/dt was similar for the two genotypes. WT muscles performed significantly more isometric work (i.e. FTI) than skMLCK$^{-/-}$ muscles (17.01±1.7 and 10.21±0.8 mN s, respectively, P<0.01) during the protocol.

Effects of BTS during repetitive stimulation in WT and skMLCK$^{-/-}$ muscles

An 80 min incubation in 25 µmol l^{-1} BTS abolished isometric twitch force of WT and skMLCK$^{-/-}$ muscles similarly (by 94±1% and 95±1%, respectively). Changes to work (as FTI) of WT and skMLCK$^{-/-}$ muscles were also similar (by 88±2% and 90±1%, respectively) (Fig. 3).

Myosin RLC phosphorylation in WT and skMLCK$^{-/-}$ muscles

At rest, WT muscles had 11-fold more RLC phosphorylation than skMLCK$^{-/-}$ muscles (i.e. 0.22±0.02 and 0.02±0.01 mol phosphate mol^{-1} RLC, respectively) (Fig. 4). In WT muscles frozen immediately after repetitive stimulation, RLC phosphorylation was increased ~3.5-fold but was not changed in skMLCK$^{-/-}$ muscles (to 0.76±0.02 and 0.02±0.01 mol phosphate mol^{-1} RLC,

Table 1. Isometric twitch characteristics of WT and skMLCK$^{-/-}$ muscles during repetitive stimulation

	Initial twitch	25th	50th	150th
P_t (mN)				
WT	45.8±3.5^{a}	45.3±3.2^{a}	49.7±3.0^{b}	56.3±2.4^{c}
skMLCK$^{-/-}$	40.2±2.8^{a,c}	37.5±2.7^{b*}	38.9±2.8^{ab*}	42.2±3.0^{c*}
TPT (ms)				
WT	18.3±0.3^{a}	16.3±0.3^{b}	16.0±0.3^{c}	15.8±0.3^{c}
skMLCK$^{-/-}$	17.3±0.4^{a,*}	15.6±0.2^{b,*}	14.9±0.3^{c,*}	14.4±0.4^{c,*}
$RT_{1/2}$ (ms)				
WT	12.1±0.4^{a}	8.1±0.2^{b}	7.6±0.3^{b}	9.6±0.5^{c}
skMLCK$^{-/-}$	10.9±0.3^{a,*}	7.6±0.3^{b}	6.9±0.2^{b}	7.3±0.2^{b,*}
+dP/dt (mN ms^{-1})				
WT	2.5±0.2^{a}	2.7±0.2^{b}	3.1±0.2^{c}	3.6±0.2^{d}
skMLCK$^{-/-}$	2.3±0.1^{a}	2.4±0.2^{a}	2.6±0.2^{b*}	2.9±0.2^{c*}
−dP/dt (mN ms^{-1})				
WT	1.9±0.2^{a}	2.8±0.2^{b}	3.3±0.1^{c}	3.0±0.1^{b}
skMLCK$^{-/-}$	1.9±0.2^{a}	2.5±0.2^{b}	2.9±0.2^{c}	2.8±0.2^{c}

Values are means±s.e.m.; n=8. Muscles were stimulated at 10 Hz for 15 s at a muscle length of 0.9 optimal length (L_o). WT, wild-type; skMLCK$^{-/-}$, skeletal myosin light chain kinase ablated; P_t, peak isometric twitch force; TPT, time to peak tension; $RT_{1/2}$, half-relaxation time; +dP/dt, rate of force development; −dP/dt, rate of force relaxation. Means with different letters are significantly different within row (within genotype; Sidak's correction, P<0.05); *skMLCK$^{-/-}$ is significantly different from WT at that time point. A repeated measures ANOVA was run for each genotype to compare the various time points, while unpaired t-tests compared the two genotypes at each individual time point.

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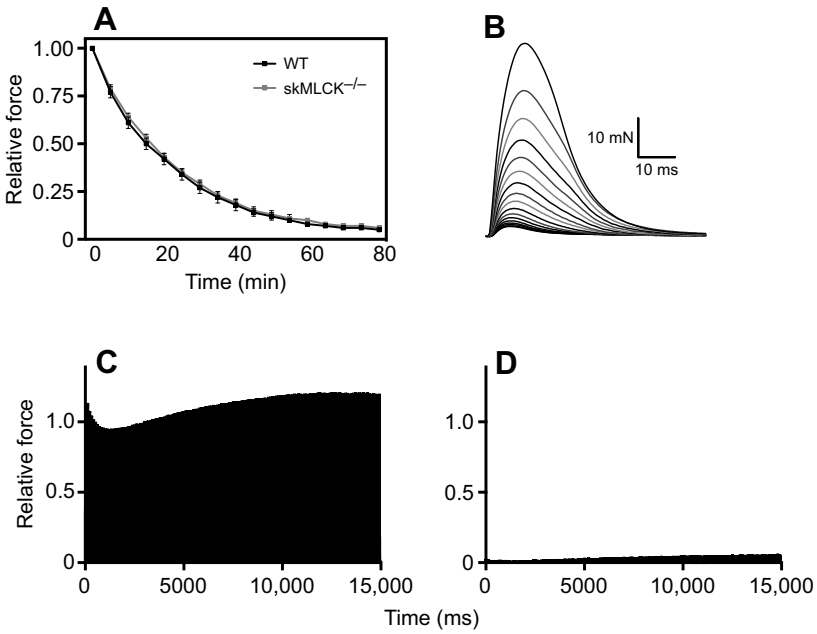

Fig. 3. Summary of effect of 25 µmol l^{-1} BTS on isometric twitch force of WT and skMLCK$^{-/-}$ muscles (*in vitro*, 25°C). (A) Time course of relative force inhibition for WT and skMLCK$^{-/-}$ muscles over an 80 min incubation period. Values are means±s.e.m. for each group (n=7–8). (B) Representative traces obtained 5 min apart during *N*-benzyl-*p*-toluene sulfonamide (BTS) incubation depicting the effect on twitch amplitude and time course (only WT shown). (C) Representative WT record during repetitive low-frequency stimulation in the absence of BTS; staircase potentiation was ~20% in this example. (D) Representative WT record during repetitive low-frequency stimulation in the presence of BTS; note that despite a force depression of ~95%, staircase potentiation was still observed.

respectively). Data for muscles from control and matched BTS conditions were similar and thus were pooled for this analysis.

Effects of repetitive stimulation on metabolites in WT and skMLCK$^{-/-}$ muscles without BTS

Resting metabolite concentrations did not differ between genotypes or incubation conditions in unstimulated muscles (Table 2). In addition, no significant differences existed between genotypes or incubation conditions in concentrations of PCr, Cr or ATP following repetitive stimulation; however, WT control muscles had significantly greater lactate concentrations after stimulation than WT BTS and skMLCK$^{-/-}$ BTS muscles (Table 2).

No differences in PCr, Cr, ATP or lactate were detected between genotypes, with or without BTS, in unstimulated muscles (Table 2). Similar to the basal condition, no significant differences in PCr, Cr or ATP were found between genotypes with or without BTS following stimulation; in both genotypes, control muscles did have significantly greater lactate concentrations than BTS-treated muscles, however (Table 2).

Energy consumption and contractile economy of WT and skMLCK$^{-/-}$ muscles

Total energy consumption, partitioned into cross-bridge and activation energy consumption, is shown for both genotypes in Fig. 5. No effect of genotype was found as the E_{total} of WT and skMLCK$^{-/-}$ muscles was similar (Table 3). When E_{total} was partitioned by BTS treatment, E_A was also similar between genotypes, but E_{XB} was greater for skMLCK$^{-/-}$ than for WT muscles. Because work was increased but E_{total} was similar, the contractile economy of WT muscles was significantly greater than for skMLCK$^{-/-}$ muscles (Fig. 6).

DISCUSSION

The purpose of this study was to investigate the effects of RLC phosphorylation-mediated force potentiation on the energetic cost of contraction during low-frequency stimulation of fast twitch skeletal muscle isolated from WT and skMLCK$^{-/-}$ mice. A novel aspect of this work is that it is the first to directly examine the effects of RLC phosphorylation on muscle energetics and contractile economy by using BTS to partition energy consumption into contractile and non-contractile components. Our results in indicate that skMLCK-catalyzed increases in RLC phosphorylation augment peak isometric twitch force and work without increasing energy consumption, thus increasing low-frequency contractile economy of mouse fast twitch muscle *in vitro*.

Isometric force and work during staircase potentiation

The progressive increase in isometric force during repetitive stimulation displayed by rodent fast twitch skeletal muscle, referred to as staircase potentiation, is well documented (Close and Hoh, 1968; Krarup, 1981a; MacIntosh et al., 1993; MacIntosh and Kupsh, 1987; Smith et al., 2014). Our findings of increased force and work by WT but not skMLCK$^{-/-}$ muscles is consistent with the idea that RLC phosphorylation causes staircase potentiation. Evidence that RLC phosphorylation is the main, but not only, mechanism for this response is that muscles from skMLCK$^{-/-}$ mice display much

Table 2. Effect of BTS on metabolites of unstimulated and stimulated WT and skMLCK$^{-/-}$ muscles

	PCr	Cr	ATP	Lactate
Unstimulated muscles				
WT				
Control	93.9±1.1	17.8±1.1	21.9±0.6	2.8±0.8
BTS	94.7±1.0	17.0±1.0	23.6±0.8	2.5±0.8
skMLCK$^{-/-}$				
Control	95.6±3.0	16.1±3.0	23.4±0.6	3.1±0.8
BTS	94.1±3.4	17.6±3.4	22.0±0.6	2.5±0.5
Stimulated muscles				
WT				
Control	59.6±5.0	52.1±5.0	23.2±1.1	13.1±0.8[a]
BTS	71.1±2.3	40.6±2.3	22.1±0.9	7.2±1.1[b]
skMLCK$^{-/-}$				
Control	62.9±2.6	48.8±2.3	21.0±1.1	10.1±1.2[a,b]
BTS	71.4±1.8	40.3±1.8	22.0±1.6	6.5±1.7[b]

Values are means±s.e.m., n=7 muscles. Values are given in µmol g^{-1} dry mass. BTS, *N*-benzyl-*p*-toluene sulfonamide; PCr, phosphocreatine; Cr, creatine. Muscles were incubated for 80 min at 25°C in the absence (control) or presence of 25 µmol l^{-1} BTS. At 80 min, muscles were immediately snap-frozen and stored at –80°C until analyzed.

Journal of Experimental Biology (2018) 221, jeb167718. doi:10.1242/jeb.167718

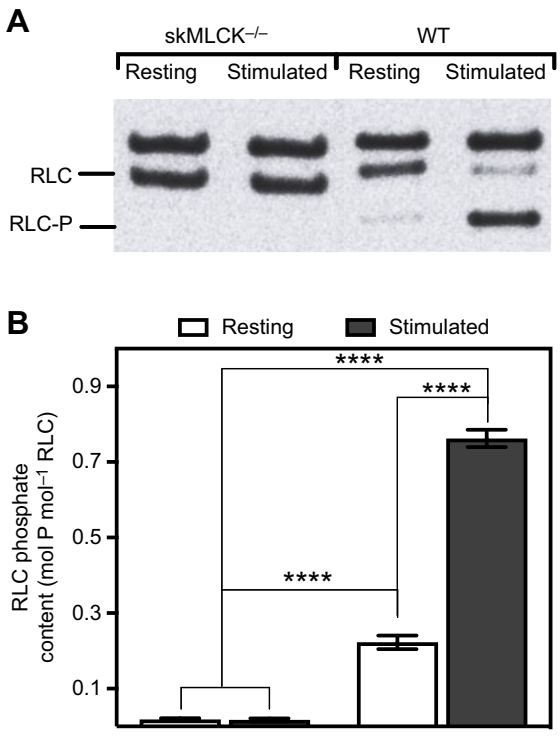

Fig. 4. Effect of skMLCK expression on myosin regulatory light chain (RLC) phosphorylation of mouse EDL muscle (*in vitro*, 25°C). (A) Representative urea–glycerol PAGE blot of non-phosphorylated and monophosphorylated myosin RLC of isolated EDL muscles from WT and skMLCK$^{-/-}$ mice. One muscle was frozen after an 80 min equilibration period (Resting) while the contralateral muscle from the same animal was frozen after 15 s of repetitive low-frequency stimulation (Stimulated). Fractional myosin phosphorylation was calculated as the ratio of the phosphorylated band to total light chain content (RLC phosphorylation=[RLC-P]/[RLC]+[RLC-P]). Note that the top bands in each lane are unidentified in each genotype. (B) Summary of effect of genotype (skMLCK$^{-/-}$ versus WT) and stimulation (resting versus stimulated) on RLC phosphorylation of mouse EDL muscle. RLC phosphorylation is expressed as mol phosphate mol^{-1} RLC. Data from BTS-untreated and -treated muscles were pooled for this analysis. Values are means±s.e.m.; n=6, ****P<0.0001 for two-way ANOVA with *post hoc* analyses conducted using Tukey's HSD.

reduced staircase potentiation compared with muscles from WT mice (Zhi et al., 2005). In addition, rat disuse or denervation models show that muscles with reduced RLC phosphorylation also display reduced staircase potentiation (MacIntosh et al., 2008; Rassier et al., 1999). The fact that the initial decrease in force observed during stimulation (i.e. negative staircase) was greater in skMLCK$^{-/-}$ than in WT muscles also accords with the idea that, without RLC phosphorylation, low-frequency forces are compromised. By contrast, changes to twitch TPT or $RT_{1/2}$ noted during staircase may be unrelated to RLC phosphorylation (Smith et al., 2014).

Myosin RLC phosphorylation

The levels of RLC phosphorylation we observed following low-frequency stimulation producing staircase potentiation are similar to those reported previously using similar stimulus protocols (MacIntosh et al., 1993) or similar muscle models (Moore et al., 1990). Consistent with previous work comparing the two genotypes, we found that RLC phosphorylation was increased by stimulation in WT but not in skMLCK$^{-/-}$ muscles (Bowslaugh et al., 2016; Gittings et al., 2011, 2016; Zhi et al., 2005). These data confirm the critical influence of skMLCK for stimulation-induced

Table 3. Total high-energy phosphate consumption of WT and skMLCK$^{-/-}$ muscles during repetitive low-frequency stimulation

	E_{total} (μmol P)	Raw E_A (μmol P)	Force inhibition (%)	E_{XB} (%)	E_A (%)
WT	47.1±6.9	33.5±3.6	88.2±1.8	32.7	67.3
skMLCK$^{-/-}$	48.1±3.1	28.6±4.6	90.4±0.8	44.9	55.1

Values are means±s.e.m., n=7. E_{total}, total energy consumption; E_A activation energy turnover; E_{XB}, crossbridge energy consumption. Raw E_A values were directly measured from BTS-treated muscles. Force inhibition during BTS incubation was then used to calculate corrected E_{XB} and E_A values.

RLC phosphorylation levels. Interestingly, because skMLCK activity is Ca^{2+} dependent (Stull et al., 2011), the fact that stimulated values for WT RLC phosphorylation were similar with or without BTS is consistent with the idea that this drug does not influence Ca^{2+} handling (Pinniger et al., 2005).

Energy consumption and economy of WT versus skMLCK$^{-/-}$ muscle

The ATP cost of skMLCK-catalyzed RLC phosphorylation has been estimated to be ~5% of total energy used during a 5 s tetanus (at 0°C) (Homsher, 1987). After correcting for residual force development during BTS incubation, no differences in E_{total} or E_A were found between genotypes in the present study (Fig. 5). This suggests that the energetic cost of skMLCK-catalyzed RLC phosphorylation was negligible and below the detection limit of our assays. This may be due to the very slow kinetics of the PP1 phosphatase enzyme which greatly reduces the skMLCK activity required to maintain RLC phosphorylation levels during stimulation (Stull et al., 2011).

An important assumption in the present study is that the genotype-dependent difference in contractile economy we observed was due to the presence of skMLCK-catalyzed phosphorylation of the RLC in WT but not skMLCK$^{-/-}$ muscles. Although the precise mechanism for this putative effect of RLC phosphorylation cannot be identified by our data, work on single rabbit myosin molecules indicates that RLC phosphorylation does not directly influence myosin ATPase rate (Morgan et al., 1976). In accord with this, genotype-dependent differences in potentiation due to phosphorylation-mediated increases in the number of attached cross-bridges should produce a commensurate increase in E_{XB} (see fig. 2 of Sweeney and Stull, 1990). Thus, a working

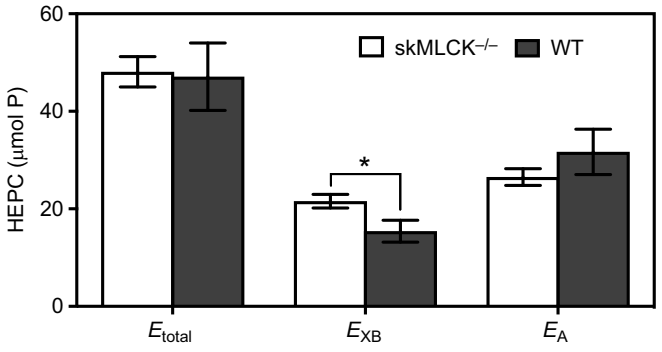

Fig. 5. High-energy phosphate consumption (HEPC) of WT and skMLCK$^{-/-}$ muscles during repetitive low-frequency stimulation (*in vitro*, 25°C). Bars show total energy (E_{TOTAL}), cross-bridge (E_{XB}) and activation (E_A) energy components for each genotype when corrected for residual force production in the presence of BTS. All values are means±s.e.m. (n=7, contralateral pairs). *P<0.05 for two-tailed unpaired t-test. See Materials and methods for details.

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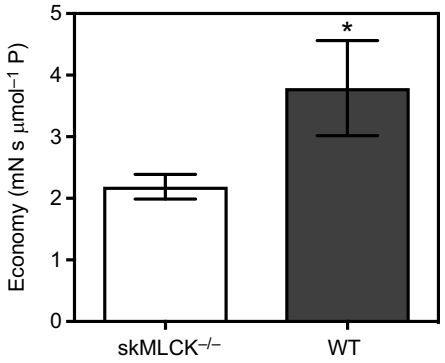

Fig. 6. Contractile economy of WT and skMLCK$^{-/-}$ muscles during repetitive low-frequency stimulation (*in vitro*, 25°C). Values are expressed as means±s.e.m. (n=7). *P<0.05 for two-tailed unpaired t-test with Welch's correction.

hypothesis for our results is that, in addition to a myosin phosphorylation-mediated increase in the portion of cycling cross-bridges able to participate in isometric force generation, there may have also been an increase in the force generated per attached cross-bridge. Results from studies on single myosin motor molecules from rabbit psoas muscle may provide clues as to how this unitary property may have been altered by RLC phosphorylation. For example, Greenberg et al. (2009) showed that phosphorylated cross-bridges detach more slowly than unphosphorylated cross-bridges, thus increasing cross-bridge duty cycle (cf. Duggal et al., 2014). Alternatively, Greenberg et al. (2010) showed that phosphorylation may increase the rigidity of the light chain-binding domain, thus increasing cross-bridge stiffness. In principle, either of these molecular outcomes could increase the thermodynamic efficiency of the cross-bridge force-generating cycle by increasing the life time of the force-generating state or by increasing the force exerted per force-generating cross-bridge, respectively. However, a phosphorylation-mediated slowing of cross-bridge detachment rate is incompatible with results from intact muscle showing no change in unloaded shortening velocity, a mechanical surrogate for cross-bridge detachment kinetics, between WT and skMLCK$^{-/-}$ or between phosphorylated and unphosphorylated muscles (Barsotti and Butler, 1984; Butler et al., 1983; Gittings et al., 2011; Palmer and Moore, 1989). Thus, we favor the idea that the disproportionate increase in cross-bridge force and work to E_{XB} we found was due to stiffness-mediated increases in force per attached cross-bridge.

Relationship with pertinent work

Our data are consistent with results from mouse EDL muscle showing stimulation duration-dependent reductions in the average rate of energy consumption (Barsotti and Butler, 1984). They also accord with the results of Crow and Kushmerick (1982), who reported a correlation between RLC phosphorylation and reduced energy cost for isometric tetani in mouse EDL muscle. However, our findings are in stark contrast to those from rat skeletal muscle *in situ* showing that RLC phosphorylation increased the energetic cost of high-frequency, isovelocity contractions to a higher extent than it increased work output, thus decreasing economy (Abbate et al., 2001). Although isometric twitch force potentiation magnitude increases with temperature in the range 25–35°C (Krarup, 1981b; Moore et al., 1990), cross-bridge efficiency of mammalian muscle has been shown to be constant between 25 and 30°C (Barclay et al., 2010). Thus, excluding the possibility of a steep change at temperatures just above 30°C, we suggest that differences between our study and that of Abbate et al. (2001) are due to stimulation frequency and/or contraction-type dependencies for the influence of RLC phosphorylation on force (e.g. Gittings et al., 2012). For example, low-frequency stimulation resulting in low levels of thin filament activation may result in lower work performed per cycling cross-bridge compared with the effects of stimulation at high frequencies (Lewis and Barclay, 2014). Thus, our staircase protocol may have preferentially enhanced contractile economy by amplifying the structure–function influence of RLC phosphorylation on cross-bridge stiffness and/or duty cycle compared with the tetanic protocol used by Abbate et al. (2001). Alternatively, the increase in cross-bridge efficiency displayed by rodent skeletal muscle at moderate compared with slow or isometric speeds of shortening (see fig. 11 of Barclay, 2015) may have negatively biased the ability of the RLC phosphorylation mechanism to further enhance efficiency. More work is needed to distinguish between these possibilities.

BTS experiments

Consistent with previous work on rat skeletal muscle, our BTS experiments revealed a relatively low contribution of E_{XB} to E_{total} in either genotype (~40%) (Siegman et al., 1994; Zhang et al., 2006). Although BTS is a selective inhibitor of myosin II ATPase activity (Bruton et al., 2006; Pinniger et al., 2005), this may be accounted for on the basis of temperature as SERCA ATPase activity has been shown to comprise ~50% of total ATP consumption at 20°C in rat muscle (Rall, 1982; Stienen et al., 1995). Alternatively, because mouse EDL muscle consists predominately of IIX and IIB fibers (Smith et al., 2013), this profile may have contributed to a relatively high E_A versus E_{XB} compared with other muscle models with more heterogeneous fiber-type composition. Despite our finding that E_A accounts for a high portion of E_{total} during repetitive low-frequency stimulation of mouse EDL (Barclay and Loiselle, 2007), the E_{XB} is still large enough for a myosin phosphorylation-mediated influence on muscle energetics.

Summary

This study provides evidence that RLC phosphorylation-mediated force potentiation improves the contractile economy of fast twitch mammalian muscle during low-frequency, repetitive stimulation of mouse EDL muscle *in vitro*. Use of skMLCK$^{-/-}$ muscles provided a negative control allowing us to show that the energetic overhead for skMLCK-catalyzed phosphorylation of the RLC is insignificant compared with the overall energy cost, and that RLC phosphorylation enhances the thermodynamic efficiency of the cross-bridge force-generating cycle. Finally, isometric twitch potentiation is length dependent (Rassier and MacIntosh, 2002) and our experiments were performed at a length (0.90 L_o) intended to maximize this outcome. It thus remains to be shown whether experiments performed at longer lengths or those involving length excursions display the same changes to contractile economy as observed in the present experiments.

Acknowledgements

The authors would like to acknowledge the support of Dr J. Stull (Department of Physiology, University of Texas Southwestern Medical Center) in development of the skMLCK$^{-/-}$ mouse colony and with methods for quantification of myosin RLC phosphate content.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.B., W.G., R.V.; Methodology: J.B., W.G., R.V.; Formal analysis: J.B.; Investigation: J.B., W.G.; Resources: R.V.; Writing - original draft: J.B.; Writing -

review & editing: R.V., J.B., W.G.; Supervision: R.V.; Project administration: R.V.; Funding acquisition: R.V.

Funding
This research was made possible by funding from the Natural Sciences and Engineering Council of Canada (RV Discovery Grant: 2014-05122).

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on request.

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