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Fast skeletal muscle troponin activator CK-2066260 increases fatigue resistance by reducing the energetic cost of muscle contraction

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Key points

- Skeletal muscle fatigue limits performance in various physical activities, with exercise intolerance being a key symptom in a broad spectrum of diseases.
- We investigated whether a small molecule fast skeletal troponin activator (FSTA), CK-2066260, can mitigate muscle fatigue by reducing the cytosolic free $[Ca^{2+}]$ required to produce a given submaximal force and hence decreasing the energy requirement.
- Isolated intact single mouse muscle fibres and rat muscles *in-situ* treated with CK-2066260 showed improved muscle endurance., which was accompanied by decreased ATP demand and reduced glycogen usage.
- CK-2066260 treatment improved *in-vivo* exercise capacity in healthy rats and in a rat model of peripheral artery insufficiency.
- In conclusion, we show that the FSTA CK-2066260 effectively counteracts muscle fatigue in rodent skeletal muscle *in vitro, in situ,* and *in vivo.* This may translate to humans and provide a promising pharmacological treatment to patients suffering from severe muscle weakness and exercise intolerance.

Abstract Skeletal muscle fatigue limits performance during physical exercise and exacerbated muscle fatigue is a prominent symptom among a broad spectrum of diseases. The present study investigated whether skeletal muscle fatigue is affected by the fast skeletal muscle troponin activator

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(FSTA) CK-2066260, which increases myofibrillar Ca^{2+} sensitivity and amplifies the submaximal force response. Because more force is produced for a given Ca^{2+}, we hypothesized that CK-2066260 could mitigate muscle fatigue by reducing the energetic cost of muscle activation. Isolated single mouse muscle fibres were fatigued by 100 repeated 350 ms contractions while measuring force and the cytosolic free $[Ca^{2+}]$ or $[Mg^{2+}]$ ($[Mg^{2+}]_i$). When starting fatiguing stimulation at matching forces (i.e. lower stimulation frequency with CK-2066260): force was decreased by ∼50% with and by ∼75% without CK-2066260; $[Mg^{2+}]_i$ was increased by ∼10% with and ∼32% without CK-2066260, reflecting a larger decrease in [ATP] in the latter. The glycogen content in *in situ* stimulated rat muscles fatigued by repeated contractions at matching forces was about two times higher with than without CK-2066260. Voluntary exercise capacity, assessed by rats performing rotarod exercise and treadmill running, was improved in the presence of CK-2066260. CK-2066260 treatment also increased skeletal muscle fatigue resistance and exercise performance in a rat model of peripheral artery insufficiency. In conclusion, we demonstrate that the FSTA CK-2066260 mitigates skeletal muscle fatigue by reducing the metabolic cost of force generation.

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Introduction

The performance during numerous physical activities is limited by a reversible decline in muscle force and performance, i.e. the development of skeletal muscle fatigue. Accelerated fatigue development is a prominent symptom among a broad spectrum of neuromuscular diseases (Petty *et al.* 1986; Drachman, 1994; Sharma *et al.* 1995; Allen *et al.* 2016). Moreover, exacerbated muscle fatigue is a frequent symptom in other diseases including cancer cachexia (Ahlberg *et al.* 2003), a side effect of pharmacological treatments, including cholesterol-reducing statins (Dirks & Jones, 2006) and a recurrent problem accompanying normal ageing (Andersson *et al.* 2011; Hepple & Rice, 2016). At the cellular level, Ca^{2+} regulation has a central role in muscle fatigue via two mechanisms (Allen *et al.* 2008). First, reductions in force are associated with decreased sarcoplasmic reticulum (SR) Ca^{2+} release, which reduces cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and consequently decreases the amount of Ca^{2+} that can bind to troponin C and enable myosin and actin interactions. Second, reduced Ca^{2+} sensitivity of the troponin–tropomyosin interaction (i.e. reduced myofibrillar Ca^{2+} sensitivity) decreases the availability of open binding sites on actin for myosin at sub-saturating $[Ca^{2+}]_i$.

In addition to its direct effect on muscle contraction, intracellular Ca^{2+} cycling requires energy and thereby may contribute to muscle fatigue. In fact, Ca^{2+} re-uptake by sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) accounts for a substantial fraction of the energy consumed in contracting muscle (Szentesi *et al.* 2001; Zhang *et al.* 2006; Barclay *et al.* 2007). Recently developed small molecule fast skeletal muscle troponin activators (FSTAs) increase myofibrillar Ca^{2+} sensitivity specifically in fast skeletal muscle fibres by prolonging the attachment of Ca^{2+} to fast skeletal muscle troponin C, leading to increased contractile force at non-saturating $[Ca^{2+}]_i$ (Russell *et al.* 2012; Hwee *et al.* 2015, 2017). Because FSTAs allow submaximal forces to be produced at a lower $[Ca^{2+}]_i$, the energetic cost to sustain a given force would hypothetically be lower due to less Ca^{2+} being released from the SR, thereby reducing the ATP needed by SERCA to pump Ca^{2+} back into the SR. We therefore investigated whether increased myofibrillar Ca^{2+} sensitivity by FSTA CK-2066260 could improve muscle performance and mitigate muscle fatigue in part through reductions in ATP use and muscle glycogen utilization.

Methods

Ethical approval

All experiments in mice were performed at the Karolinska Institutet and were carried out according to the guidelines laid down by the animal welfare committee at Karolinska Institutet. All experiments on mice complied with the Swedish Animal Welfare Act, the Swedish Welfare Ordinance, and applicable regulations and recommendations from Swedish authorities. The parts of the study involving mice were approved by the Stockholm North Ethical Committee on Animal Experiments (no. N19/15). Rats used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* of the Institute (7th edn, (US) National Research Council) and under the supervision and approval of the Cytokinetics and the University of Missouri Institutional Animal Care and Use Committees. All animal experiments conform to the principles and regulations as described in the editorial by Grundy (2015).

Animals

Adult (10–15 weeks) female C57BL/6J mice (Scanbur Research, Stockholm, Sweden) and adult (9–11 weeks) female and male Sprague–Dawley rats (Charles River Laboratories, Hollister, CA, USA) were used for these studies. In total we used 20 mice and 105 rats. Animals were housed in a temperature-controlled environment with a 12 h light–dark cycle and were provided with standard rodent food and water *ad libitum*.

Single muscle fibre experiments

Mice were killed by cervical dislocation and whole flexor digitorum brevis (FDB) muscles from the hindleg were removed. Intact single FDB muscle fibres with attached tendons were mechanically dissected from whole FDBs, and then mounted between an Akers 801 force transducer (Kronex Technologies, Oakland, CA, USA) and an adjustable holder (Cheng & Westerblad, 2017). Fibres were continually perfused with room temperature (~26°C) Tyrode solution containing (in mM): 121 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.4 NaH_2PO_4, 24.0 $NaHCO_3$, 0.1 EDTA, and 5.5 glucose. The solution was bubbled with 95% O_2–5% CO_2, giving a bath pH of 7.4. Fetal calf serum (0.2%) was added to the solution. Platinum foil electrodes placed longitudinally on both sides of the fibre were used to deliver electrical current pulses, and fibres were stimulated with 350 ms duration 70 Hz tetani (at 0.5 ms pulse width) to adjust to the optimal length of force production. Fibre cross-sectional area was measured at this length to calculate force per cross-sectional area. $[Ca^{2+}]_i$ was measured with the fluorescent indicator indo-1 (Thermo Fisher Scientific, Stockholm, Sweden), which was pressure injected into the fibre using a micropipette. Indo-1 was excited at 360 ± 5 nm and the emitted fluorescent light was recorded at 405 ± 5 nm and 495 ± 5 nm using a system consisting of a xenon lamp, a monochromator and two photomultiplier tubes (Horiba, London, ON, Canada). Ratiometric indo-1 emission values were converted to $[Ca^{2+}]_i$ using an *in vivo* calibration as reported previously (Cheng & Westerblad, 2017).

Fatigue was induced by repetitively stimulating the fibres with 350 ms duration 70 Hz tetani every 5 s until force decreased to 40% of the initial value. At this point, repetitive stimulation continued and fibres were superfused with Tyrode solution containing either the fast skeletal muscle troponin activator CK-2066260 (10 μM) (Cytokinetics, San Francisco, CA, USA), or caffeine (5 mM). The concentration of CK-2066260 (10 μM) was chosen based on our previous results showing that this concentration effectively and reversibly increases submaximal force while having no significant effect on $[Ca^{2+}]_i$ at rest or during contractions (Hwee *et al.* 2017).

In some experiments, we assessed whether CK-2066260 could improve fatigue resistance in fibres where initial peak force was matched to that of untreated fibres. Fibres were then superfused with CK-2066260 for 5 min before repetitive stimulation and throughout the repetitive stimulation. At the start of repetitive stimulation, both CK-2066260 treated and non-treated fibres were stimulated at a frequency achieving 80% of maximum tetanic force. Additional experiments were performed in which CK-2066260 treated and non-treated fibres were repeatedly stimulated at 120 Hz giving near maximum tetanic force and $[Ca^{2+}]_i$.

Mg^{2+} binds tightly to ATP and the cytosolic free Mg^{2+} concentration ($[Mg^{2+}]_i$) therefore increases when [ATP] decreases (Westerblad & Allen, 1992; Leyssens *et al.* 1996; Dahlstedt & Westerblad, 2001). Thus, $[Mg^{2+}]_i$ measurements provide a relatively easy way to follow changes in [ATP] during induction of fatigue in single muscle fibres; the results obtained with this method are similar to those obtained when [ATP] was measured directly using a luciferin–luciferase-based method, which is technically highly demanding and has a low success rate (Allen *et al.* 2002). $[Mg^{2+}]_i$ was measured in a separate group of fibres to determine potential differences in energy metabolism between CK-2066260 treated and non-treated fibres. Fibres were pressure injected with the fluorescent indicator mag-indo-1 (TEFLabs, Austin, TX, USA), which was excited at 343 ± 5 nm wavelength and emission was recorded at 405 ± 5 nm and 495 ± 5 nm wavelengths.

Muscle glycogen content measurement following muscle fatigue *in situ*

In situ muscle fatigue was induced by repetitive nerve stimulation in female Sprague–Dawley rats as previously described (Hwee *et al.* 2015). Rats were placed under anaesthesia with isoflurane (1–5%). An incision was made on the mid-thigh region of the right leg to expose the sciatic nerve. To prevent co-contraction of the ankle dorsiflexors, the peroneal nerve was severed and isolated. Rats were then placed on a temperature-maintained *in situ* muscle analysis rig (Model 806C; Aurora Scientific, Aurora, ON, Canada). The knee was immobilized in a clamp between two sharpened screws, and the foot was taped to a footplate attached to a force transducer (Aurora Scientific). Stainless steel needle electrodes (0.10 mm) were hooked around the exposed sciatic nerve. Rats were treated with vehicle (10% dimethylacetamide (DMA):50% polyethylene glycol (PEG):16% sulfobutylether-β-cyclodextrin (Cavitron), I.V.) or CK-2066260 (10 mg kg^{-1}, I.V.) and muscle contractile properties were assessed by applying an electrical current (under supramaximal voltage conditions) to the nerve and recording the force generated by the muscle via a servomotor. Three hundred repeated 25 Hz or 50 Hz stimulations were applied (350 ms train duration, 1 stimulation s^{-1}) and the resulting force was recorded.

At the end of the protocol, the medial gastrocnemius was rapidly harvested and frozen in isopentane.

The glycogen content was assessed on 10 μm-thick medial gastrocnemius muscle cross-sections using a periodic acid–Schiff stain (Thompson, 1966; Sheehan & Hrapchak, 1987). Digital images were obtained under 200× total magnification (Olympus BX41; Olympus America, Melville, NY, USA). Glycogen content was quantified in a blinded fashion by applying a uniform pixel colour threshold to identify the percentage of muscle region that was positively stained for glycogen.

In vivo functional assessment in healthy rats

Voluntary functional performance was assessed in female rats by rotarod exercise (Model No. 2325-0205, San Diego Instruments, San Diego, CA, USA) and by treadmill running (model Exer 3/6, Columbus Instruments, Columbus, OH, USA). All rats in the rotarod experiments were acclimated to the rotarod the day before the experimental session. Acclimation consisted of placing the rats on the rotating drum that revolved at a constant 10 r.p.m. speed for 5 min. After all rats were subjected to the 5 min trial, a second training session was initiated with a progressively increasing speed from 14 to 16 r.p.m. over a 5 min period. Rats that failed to run during the course of the training were excluded from the experiment. On the day of testing, vehicle (0.2% Tween-80, 1% hydroxypropyl methyl cellulose (HPMC)) or CK-2066260 (10 mg kg^{-1} in formulation vehicle, P.O.) was administered 30 min before exercise assessment; experimenters were blinded to the type of treatment. Rats ran at a constantly accelerating rate from 12 to 25 r.p.m. over the course of 10 min, and the time to fall was recorded.

All rats in the treadmill experiments were acclimated to the treadmill over five running periods at 25 m min^{-1} for 10 min on a 5° incline. A blinded cross-over study design was performed where rats were randomly orally dosed with vehicle (0.5% HPMC:0.2% Tween-80) or CK-2066260 (10 mg kg^{-1}, P.O.) 30 min before exercise assessment. Rats ran on the treadmill at 35 m min^{-1} on a 5° incline until exhaustion. Two days later, rats received the opposite treatment and treadmill performance was assessed again.

Acute femoral artery ligation *in situ* force measurements

Femoral artery ligation (FAL) was performed in anaesthetized female Sprague–Dawley rats to induce rapid fatigue in extensor digitorum longus (EDL) muscles subjected to repetitive peroneal nerve stimulation. Rats were anaesthetized with inhaled isoflurane (1–5%). An incision was made in the thigh region and the femoral artery was isolated and dissected free of connective tissue. Multiple loose knots of 4-0 suture were placed around the artery, as previously described (Challiss *et al.* 1986).After I.V. delivery of vehicle (10% DMA:50% PEG:16% Cavitron) or CK-2066260 (2 mg kg^{-1}, I.V.), ligation was completed by tightening the knots. Repetitive nerve stimulations were performed (350 ms duration, 1 stimulation $(3\ s)^{-1}$) at a frequency that produced the same initial tension in both groups (Veh: 30 Hz; CK-2066260: 26 Hz). Force was continuously measured over the 10 min repetitive stimulation period.

Chronic femoral artery ligation treadmill performance

Bilateral FAL was performed as previously described (Yang & Terjung, 1993; Yang *et al.* 1995*a*,*b*). Male rats were anaesthetized with ketamine–acepromazine (100 mg kg^{-1}–0.5 mg kg^{-1}), and the femoral arteries were isolated just distal to the inguinal ligament. A ligature was placed tightly around each femoral artery. Ligation of the femoral artery created peripheral arterial insufficiency, in which the capacity for hindlimb blood flow is significantly reduced. Both femoral arteries were occluded by a surgical procedure that was brief, achieved with a 100% success rate, and permitted rapid recovery of the animal. Two weeks after FAL, animals were treated with vehicle (0.5% HPMC:0.2% Tween-80) or CK-2066260 (10 mg kg^{-1}, P.O.) in a blinded manner and treadmill performance was assessed as previously described (Yang *et al.* 1995*b*; Prior *et al.* 2004). On a 15° incline, rats ran at a speed of 15 m min^{-1} for 10 min, 20 m min^{-1} for 10 min, and 25 m min^{-1} until exhaustion. Treadmill running time was recorded.

Statistics

Data are presented as the mean ± SEM. Paired and unpaired Student's *t* tests, one-way ANOVA and one-way repeated measures ANOVA were used to determine statistically significant differences as appropriate with Sigmaplot 13.0 (Systat Software Inc., San Jose, CA, USA) or Prism 8 (GraphPad Software, San Diego, CA, USA). Tukey's or Dunnett's tests were used for *post hoc* analyses when significant differences were determined from ANOVA. The level of significance was set at $P < 0.05$.

Results

CK-2066260 reduces muscle fatigability by increasing myofibrillar Ca^{2+} sensitivity

Intact single fibres of mouse flexor digitorum brevis (FDB) muscles were fatigued by repetitive tetanic stimulations (350 ms duration, 70 Hz) given at 5 s intervals until force decreased to 40% of the initial force. CK-2066260 (10 μM) was then applied while stimulation continued. Application of CK-2066260 resulted in a rapid force

increase that was sustained throughout the exposure period (Fig. 1*A*). Selected contractions show that the CK-2066260-induced increase in tetanic force was actually accompanied by a slight decrease in tetanic $[Ca^{2+}]_i$ (Fig. 1*B*). Thus, CK-2066260 increased force under fatigue-inducing conditions by facilitating Ca^{2+}-binding to troponin C and hence increasing the myofibrillar Ca^{2+} sensitivity.

An alternative way to counteract the Ca^{2+}-dependent decrease in force with fatigue is to increase SR Ca^{2+} release, which can be achieved by application of caffeine (Westerblad & Allen, 1991). Application of caffeine (5 mM) caused a rapid but transient increase in force (Fig. 1*C*). Selected contractions show that the initial force increase with caffeine was due to increased tetanic $[Ca^{2+}]_i$, and thereafter $[Ca^{2+}]_i$ rapidly decreased in parallel with the decrease in force (Fig. 1*D*).

Although via different mechanisms, the application of CK-2066260 and caffeine during fatiguing stimulations produced a similar initial increase in force production: tetanic $[Ca^{2+}]_i$ showed a statistically significant decrease with CK-2066260 and the force-potentiating effect was due to increased myofibrillar Ca^{2+} sensitivity, whereas increased force with caffeine was due to facilitated SR Ca^{2+} release (Fig. 1*E*). After the initial force increase, the force-potentiating effect of CK-2066260 was sustained. Conversely, the caffeine-induced force increase was transient and prolonged exposure to caffeine ultimately exaggerated the fatigue-induced force decline. Moreover, CK-2066260 had no notable effect on $[Ca^{2+}]_i$ between contractions (from 102 ± 14 to 107 ± 23 nM, $P = 0.128$ paired t test, $n = 4$), whereas caffeine led to a twofold increase in $[Ca^{2+}]_i$ between contractions from 102 ± 18 to 220 ± 33 nM ($P < 0.001$ paired t test, $n = 6$). The increase in resting $[Ca^{2+}]_i$ during prolonged caffeine exposure coincided with a decrease in tetanic $[Ca^{2+}]_i$. Thus, the caffeine-induced facilitation of SR Ca^{2+} release appeared to cause SR Ca^{2+} unloading and transfer of Ca^{2+} into the myoplasm, which resulted in severely decreased tetanic $[Ca^{2+}]_i$ accompanied by markedly increased resting $[Ca^{2+}]_i$, although not reaching contracture levels.

CK-2066260 increases fatigue resistance of isolated muscle fibres by lowering the energetic cost of contraction

Although generally related to a high energy demand and dependency on anaerobic metabolism, the cellular mechanisms responsible for the acutely developing fatigue-induced decrease in SR Ca^{2+} release are not fully

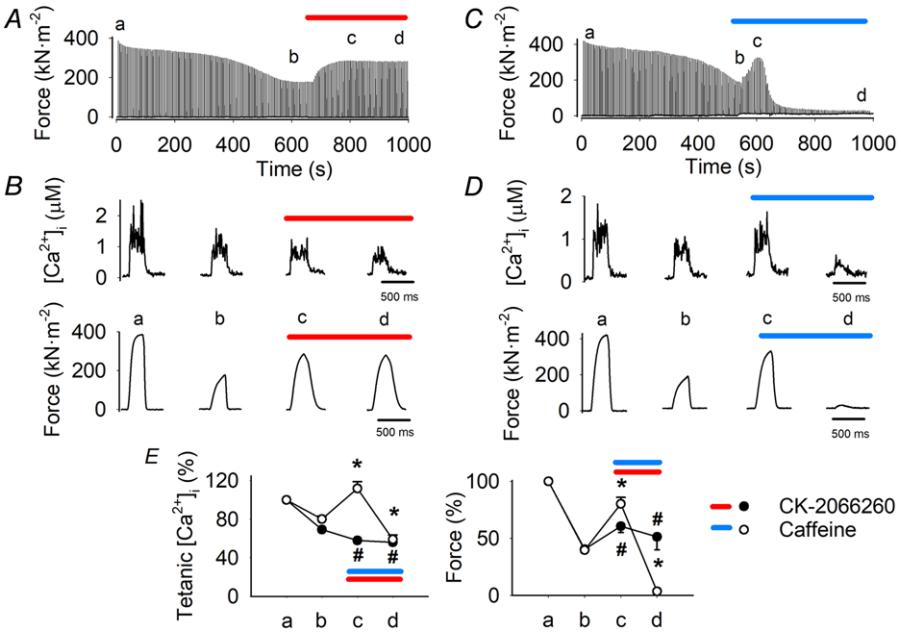

Figure 1. The myofibrillar Ca^{2+} sensitizer CK-2066260 counteracts the fatigue-induced force loss
Representative force records from single FDB fibres during repeated tetanic stimulation (70 Hz, 350 ms given at 5 s interval) with addition of either CK-2066260 (*A*) or caffeine (*C*) at the times indicated by the horizontal bars. *B* and *D*, force and $[Ca^{2+}]_i$ in the first tetanus (a), when force was decreased to 40% of the initial (b), early (c) and end (d) of exposure to CK-2066260 or caffeine, respectively. *E*, mean data (±SEM) show that CK-2066260 superfusion resulted in a sustained decrease in tetanic $[Ca^{2+}]_i$ and an increase in force (filled circles, $n = 3$–4 fibres from 4 mice), whereas caffeine induced a transient increase followed by a decrease of both tetanic $[Ca^{2+}]_i$ and force (open circles, $n = 5$–6 fibres from 3 mice). # and *, $P < 0.05$ *vs.* the value immediately before application of CK-2066260 or caffeine, respectively; one-way repeated measures ANOVA.

understood (Allen *et al.* 2008). While caffeine countered the fatigue-induced reduction in force by increasing SR Ca^{2+} release, the effect was transient, and the potentiated SR Ca^{2+} release would result in higher SERCA ATP consumption and hence an increased metabolic stress to the muscle fibre. In contrast to the effect of caffeine, the sustained force increase after CK-2066260 application was due to increased myofibrillar Ca^{2+} sensitivity, thereby avoiding increased SERCA ATP consumption. To experimentally test the link between decreased ATP utilization in the presence of CK-2066260 and improved fatigue resistance, muscle fibres were fatigued in the presence or absence of CK-2066260 with the initial force set to the same level, i.e. ~80% of maximum tetanic force. This force level was achieved at a markedly lower stimulation frequency in the presence of CK-2066260 (24 ± 5 Hz, $n = 5$ *vs.* DMSO: 50 ± 6 Hz, $n = 5$), which resulted in significantly lower $[Ca^{2+}]_i$ (0.55 ± 0.10 *vs.* 0.98 ± 0.15 μM; $P = 0.042$, unpaired *t* test). During repetitive stimulation, force was better maintained in the presence of CK-2066260 (Fig. 2*A*); after 100 fatiguing contractions, force was decreased by 49 ± 5% with CK-2066260 compared to

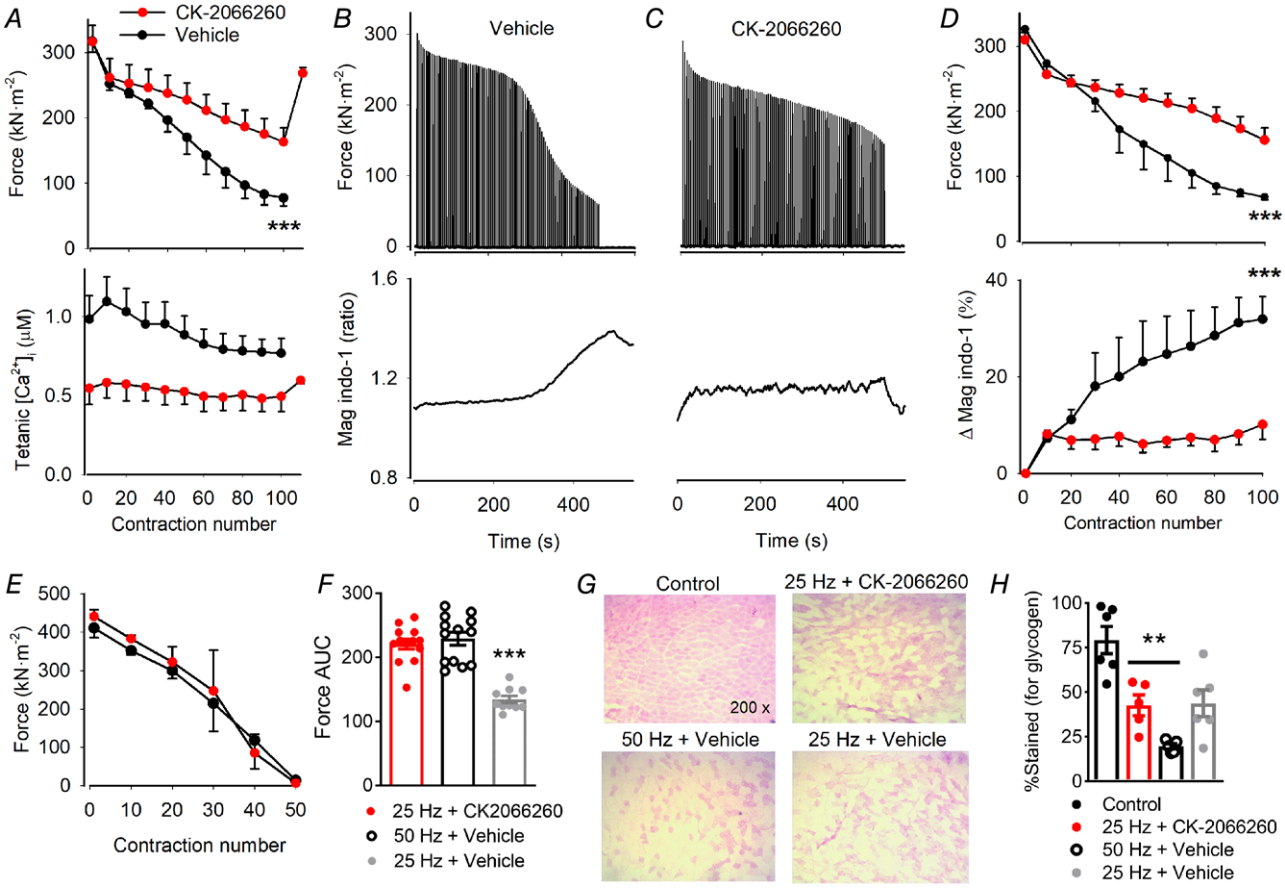

Figure 2. CK-2066260 reduces the energy utilization and increases endurance during repeated tetanic contractions
A, peak force and tetanic $[Ca^{2+}]_i$ show better endurance in CK-2066260-treated (red symbols) *vs.* untreated control (black symbols) fibres when starting at the same force ($n = 5$ fibres per group from 5 mice). Increased force and $[Ca^{2+}]_i$ after the 100th tetanus in CK-2066260-treated fibres was due to increasing the frequency to 50 Hz. Representative force and mag-indo-1 records during stimulation with matched initial forces obtained from a control (*B*) and a CK-2066260-treated (*C*) FDB fibre, and mean data from these experiments (*D*, $n = 5$ fibres per group from 4 mice). *E*, CK-2066260-treated and untreated FDB fibres showed similar declines in force during stimulation at maximal force ($n = 3$ fibres per group from 3 mice). *F*, rat hindlimb muscles treated with CK-2066260 required lower stimulation frequency (25 Hz, $n = 13$) than muscles of vehicle-treated rats (50 Hz, $n = 13$) to achieve equivalent forces during repeated tetanic stimulation (i.e. area under the curve, AUC), whereas forces of vehicle-treated rats were lower with 25 Hz stimulation ($n = 11$). Representative PAS-stained cross-sections of medial gastrocnemius muscles (*G*) and percentage of positively PAS-stained area (*H*) of unstimulated control muscles ($n = 6$), muscles of CK-2066260-treated rats stimulated with 25 Hz tetani ($n = 5$), and vehicle-treated rats stimulated with 50 Hz tetani ($n = 5$) and 25 Hz tetani ($n = 6$). Data are means ± SEM, with individual data points shown in *F* and *H*. $^{**}P < 0.01$, $^{***}P < 0.001$, CK-2066260-treated *vs.* vehicle-treated stimulated muscles with unpaired *t* test (*A* and *D*) or one-way ANOVA (*F* and *H*).

75 ± 4% without ($P < 0.001$ unpaired t test, $n = 5$ per group). Moreover, a major increase in tetanic force and $[Ca^{2+}]_i$ was obtained when the stimulation frequency of fatigued CK-2066260-treated fibres was increased to that of untreated fibres (Fig. 2*A*).

Measurements of $[Mg^{2+}]_i$ during fatigue can be used as a surrogate marker for changes in the intracellular ATP level, as ATP binds Mg^{2+} more strongly than its breakdown products (Westerblad & Allen, 1992; Leyssens *et al.* 1996; Dahlstedt & Westerblad, 2001). To further test for a potential difference in ATP utilization in control *vs.* CK-2066260-treated fibres during repetitive contractions, the above-described experiments with fatigue produced at matching initial forces were repeated while $[Mg^{2+}]_i$ was measured with the fluorescent Mg^{2+} indicator mag-indo-1 (Fig. 2*B* and *C*). Force was better maintained in the presence (decreased by 50 ± 5%, $n = 5$) than in the absence (decreased by 79 ± 2%, $n = 5$; $P < 0.001$ in unpaired t test) of CK-2066260 (Fig. 2*D*). Along with the improved force preservation in CK-2066260-treated fibres, the increase in $[Mg^{2+}]_i$ (and hence decrease in [ATP]) at the end of fatiguing stimulation was lower in these fibres than in control fibres (10 ± 3% *vs.* 32 ± 5% increase in mag-indo-1 fluorescence ratio; $P = 0.0005$, unpaired t test). These results further strengthened the hypothesis that when the stimulation frequency is set to match initial force production, the energy requirement during repeated contractions was smaller in CK-2066260-treated fibres than in control fibres.

Additional experiments were performed with fibres repetitively stimulated by high-frequency (120 Hz) tetani, where $[Ca^{2+}]_i$ is high enough to maximally activate the contractile machinery (Hwee *et al.* 2017), and the fatigue-mitigating effect of CK-2066260 would not be expected to occur because of Ca^{2+} saturation of the troponin–tropomyosin complex. As expected, the fatigue-induced force loss was similar in the untreated and CK-2066260 fibres when contractions were produced at 120 Hz (Fig. 2*E*). Thus, the improved fatigue resistance of CK-2066260-treated fibres during repeated submaximal contractions can be attributed to the increase in myofibrillar Ca^{2+} sensitivity, which reduced the $[Ca^{2+}]_i$ required for a given force and thereby decreased the energetic cost of intracellular Ca^{2+} handling.

CK-2066260 decreases the energetic cost of repeated force-matched contractions of hindlimb rat muscles activated by electrical nerve stimulation

The reduced ATP utilization with CK-2066260 after matching initial force could potentially lead to the sparing of energy substrate utilization. A major fraction of the ATP consumed during frequent, repeated muscle contraction is derived from the breakdown of intramuscular glycogen (Ørtenblad *et al.* 2013). Moreover, low glycogen availability is correlated with reduced SR Ca^{2+} release and muscle performance (Duhamel *et al.* 2006; Nielsen *et al.* 2014; Cheng *et al.* 2017). Therefore, we next tested if the ability of CK-2066260 to reduce the stimulation frequency for a given sub-tetanic force would lead to glycogen sparing. Sprague–Dawley rats were treated with vehicle or CK-2066260 (10 mg kg^{-1}, I.V.) and ankle plantar flexor muscle force was measured *in situ* in response to repeated (25 or 50 Hz) sciatic nerve stimulation. In response to 25 Hz stimulation, CK-2066260 treatment produced approximately twice the amount of force as vehicle treatment. We identified 50 Hz as a stimulation frequency with vehicle treatment that produced the same force as 10 mg kg^{-1} CK-2066260 treatment stimulated at 25 Hz (Fig. 2*F*). Compared to control (unstimulated) medial gastrocnemius muscle, all three stimulation groups had lower glycogen content as determined with periodic acid–Schiff (PAS) staining (Fig. 2*G* and *H*). At matching forces (vehicle, 50 Hz; CK-2066260, 25 Hz stimulation), muscles from CK-2066260-treated rats had significantly higher glycogen content than muscles of vehicle-treated rats ($P = 0.005$) (Fig. 2*H*). Additionally, when stimulated at 25 Hz, force was markedly lower with vehicle-treatment than with CK-2066260 treatment, but the repetitive stimulation still led to similar reductions in glycogen content in these two groups (Fig. 2*H*). To sum up, CK-2066260 reduced the sub-tetanic stimulation frequency required to produce a given target force, and thereby the energetic cost of submaximal contractions was decreased both *in vitro* and *in situ*.

CK-2066260 improves performance during voluntary endurance exercise

We next investigated the effect of CK-2066260 on *in vivo* muscle performance using either rotarod exercise or treadmill running in a blinded treatment design. Animals dosed with CK-2066260 (10 mg kg^{-1}, P.O.) ran for almost twice as long compared to vehicle treatment in the rotarod (vehicle: 4.3 ± 0.9 min ($n = 13$) *vs.* CK-2066260: 8.5 ± 1.8 min ($n = 7$), $P = 0.02$, Fig. 3*A*) and treadmill performance tests (vehicle: 63.3 ± 7.3 min ($n = 10$) *vs.* CK-2066260: 99.1 ± 9.8 min ($n = 10$), $P = 0.01$, Fig. 3*B*). We observed no side effects of CK-2066260 treatment on animal behaviour. Thus, the *in vitro* and *in situ* amplification of muscle performance and sparing of muscle energetics by CK-2066260 translated into enhanced *in vivo* physical performance in healthy animals.

CK-2066260 improves fatigue resistance in an animal model of peripheral artery insufficiency

Femoral artery ligation (FAL) is an animal model of peripheral arterial insufficiency that leads to premature fatigue

and exercise intolerance (Yang *et al.* 1995*b*; Prior *et al.* 2004). We used acute and chronic FAL models to examine the effect of CK-2066260 under impaired *in situ* and *in vivo* muscle performance conditions.

After acute induction of FAL, rats were treated with vehicle or CK-2066260 (2 mg kg^{-1}, I.V.) and the EDL muscle was subjected to repetitive peroneal nerve stimulation *in situ* for 10 min (Fig. 3*C*). Animals dosed with CK-2066260 (2 mg kg^{-1}, I.V.) produced more total force than vehicle-treated animals (vehicle: 1246 ± 104 ($n = 16$) *vs.* CK-2066260: 1713 ± 222 ($n = 8$), $P = 0.04$, Fig. 3*D*).

We followed-up the acute FAL procedure with a chronic bilateral FAL. Seven days after bilateral FAL, treadmill running time until exhaustion was 30% longer with CK-2066260 (10 mg kg^{-1}, P.O.) treatment than with vehicle treatment (17.9 ± 0.9 min ($n = 14$) *vs.* 13.9 ± 0.7 min ($n = 14$), $P = 0.002$, Fig. 3*E*). We observed no side effects of CK-2066260 treatment on animal behaviour. Overall, CK-2066260 mitigated the decline in force *in situ* and improved treadmill performance under FAL conditions.

Discussion

Exercise intolerance and premature skeletal muscle fatigue are commonly reported in various medical conditions and in normal ageing, and physical exercise can be utilized as an intervention to mitigate these impairments (Booth *et al.* 2017). However, there are situations where the ability to perform physical exercise is hampered, for instance, due to neuromuscular disorders with impaired skeletal muscle activation. Furthermore, a sedentary lifestyle and poor long-term adherence to un-supervised exercise programmes constitute general health problems and result in an increasing number of individuals with poor cardiorespiratory and muscular fitness (Booth *et al.* 2017; Byrne *et al.* 2018). Here we show that the FSTA CK-2066260 effectively counteracts fatigue development in rodent skeletal muscle *in vitro*, *in situ* and *in vivo*. Thus, FSTAs can potentially be used to improve exercise tolerance, either on their own or in combination with physical exercise.

Acute skeletal muscle fatigue, with decreased force production as a key component, develops when a large

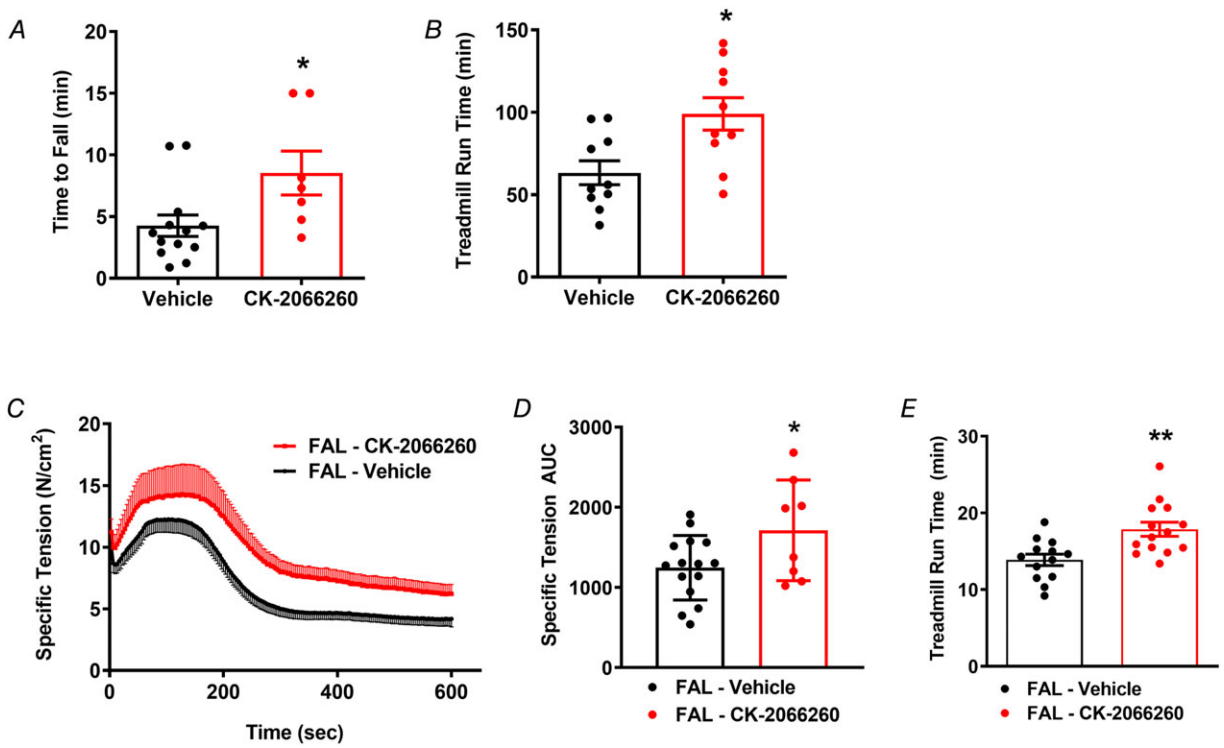

Figure 3. Effect of CK-2066260 on muscle performance in healthy rats and after femoral artery ligation (FAL)
Rotarod time to fall (vehicle-treated, $n = 13$; CK-2066260-treated, $n = 7$) (*A*) and treadmill running time until exhaustion ($n = 10$ per group) (*B*). *C*, CK-2066260 treatment increased the fatigue resistance *in situ* under FAL over a 10 min period of repeated contractions (FAL rats treated with CK-2066260, $n = 8$; vehicle-treated FAL rats, $n = 16$). *D*, accumulated force throughout repeated stimulation *in situ* (AUC, area under the curve). *E*, 2 weeks after bilateral FAL, rats treated with CK-2066260 ($n = 14$) had longer treadmill run times until exhaustion than vehicle-treated rats. Data are expressed as means ± SEM, and individual data points are shown in *A*, *B*, *D* and *E*. $^{*}P < 0.05$ and $^{**}P < 0.01$, with paired (*B*) or unpaired *t* tests (*A*, *D* and *E*).

fraction of the required energy is derived from anaerobic metabolism (Loenneke *et al.* 2014; Fan & Kayser, 2016; Cheng *et al.* 2018). During contraction of skeletal muscle fibres, energy is mainly consumed by the force-generating cross-bridges and the SR Ca^{2+}-reuptake by SERCA. CK-2066260 allows submaximal forces to be produced at a lower $[Ca^{2+}]_i$ (Hwee *et al.* 2017) and we hypothesized that this would improve muscle performance during repeated submaximal contractions because less energy is used to pump Ca^{2+} back into the SR. The results were in accordance with the hypothesis: (i) fatigue development was delayed in the presence of CK-2066260 in isolated muscle fibres, in *in situ* electrically stimulated muscle, and during rotarod and treadmill running, and (ii) utilization of ATP (as assessed by measuring $[Mg^{2+}]_i$) and glycogen was lower in the presence CK-2066260 despite similar or larger accumulated forces. Furthermore, we compared the effect of exposing fatigued muscle fibres to either CK-2066260 or caffeine (see Fig. 1). Caffeine increased force in the fatigued state by increasing SR Ca^{2+} release, which would result in increased ATP consumption both by cross-bridges and SR Ca^{2+} pumping. Thus, in general agreement with our hypothesis, the force-potentiating effect of caffeine was transient. Conversely, CK-2066260 increased force by increasing the myofibrillar Ca^{2+} sensitivity, which does not cause any additional SR Ca^{2+} pumping, and the force potentiation was sustained.

During voluntary contractions, motor units are generally activated at submaximal frequencies where the force–frequency relationship is steep (Grimby & Hannerz, 1977; Hennig & Lømo, 1985). The increase in myofibrillar Ca^{2+} sensitivity induced by FSTAs would therefore result in a relatively large increase in the force produced at the same voluntary activation level. Accordingly, treadmill running performance was improved with CK-2066260 treatment both in healthy rats and rats exposed to bilateral FAL (see Fig. 3).

CK-2066260 increases myofibrillar Ca^{2+} sensitivity by reducing the off-rate of Ca^{2+} from the Ca^{2+}-specific sites of fast skeletal muscle troponin C, hence increasing the time in its 'active' conformation (Hwee *et al.* 2017). A potentially negative effect of this is that, in addition to increasing submaximal force, CK-2066260 also decreases the rate of relaxation, which might impede performance during activities involving dynamic contractions. Still, CK-2066260 treatment resulted in improved treadmill running performance and rats treated with CK-2066260 showed improved rotarod test performance. The outcome of rotarod tests depends on neuronal control of balance, muscle coordination, and motor-planning (Karl *et al.* 2003). Thus, CK-2066260 treatment increased *in vivo* fatigue resistance without any signs of perturbed motor control and coordination, or limitations due to slowed force relaxation.

Limitations of the study

The present experiments were performed on female animals, except for the chronic FAL experiments where male rats were used. Moreover, all experiments were performed on young adult animals. Thus, additional studies on male and aged animals should be performed to evaluate potential age- and sex-related differences on rodent muscle performance with CK-2066260 treatment.

The *in situ* temperature of mouse FDB muscles is ∼31°C (Bruton *et al.* 1998) and the present experiments on single FDB muscle fibres were performed at a lower temperature (∼26°C). The force-potentiating effect of CK-2066260 has been shown to decrease with increasing temperature but with the concentration of CK-2066260 used here (10 μM), submaximal (30 Hz) force was still increased by ∼140% at 31°C, which compares to ∼250% increase at 26°C (Hwee *et al.* 2017). Thus, as judged from the large increase in submaximal force also at 31°C, an endurance-improving effect of CK-2066260 would be expected in single FDB fibre experiments performed at the *in situ* temperature.

Conclusion

We here provide *in vitro*, *in situ* and *in vivo* results demonstrating that an FSTA can reduce the energetic cost of muscle contraction and prolong performance under fatiguing conditions. In healthy human subjects, the FSTAs *tirasemtiv* and *reldesemtiv* have been shown to amplify the skeletal muscle force response to nerve stimulation (Hansen *et al.* 2014; Andrews *et al.* 2018). Thus, the performance-enhancing effects of FSTA observed in the present study may translate to humans and then provide a promising pharmacological treatment to patients suffering from severe muscle weakness and exercise intolerance.

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Additional information

Competing interests

At the time of the study, D.T.H., L.H.K., N.D., A.C.H., A.R.K., J.R.J. and F.I.M. were employees of Cytokinetics and were compensated financially for their work. The laboratory of H.W. receives financial support from Cytokinetics.

Author contributions

All authors were involved in the study design. Intact single fibre experiments were performed by A.J.C. *In situ* experiments were performed by D.T.H., L.H.K. and A.C.H. at Karolinska Institutet. *In vivo* experiments were performed by D.T.H., L.H.K., N.D., H.T.Y. and A.R.K. Aside from the chronic FAL experiments (done at the University of Missouri), all other *in situ* and *in vivo* experiments were done at Cytokinetics. The manuscript was drafted by A.J.C., D.T.H. and H.W. and all authors were involved in the revisions up to the final, submitted version. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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