Activation of fast skeletal muscle troponin as a potential therapeutic approach for treating neuromuscular diseases

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Limited neural input results in muscle weakness in neuromuscular disease because of a reduction in the density of muscle innervation, the rate of neuromuscular junction activation or the efficiency of synaptic transmission. We developed a small-molecule fast-skeletal–troponin activator, CK-2017357, as a means to increase muscle strength by amplifying the response of muscle when neural input is otherwise diminished secondary to neuromuscular disease. Binding selectively to the fast-skeletal–troponin complex, CK-2017357 slows the rate of calcium release from troponin C and sensitizes muscle to calcium. As a consequence, the frequency of nerve firing to the corresponding muscle force is amplified by binding to its troponin complex. We prepared nearly pure populations of fast, slow and cardiac myofibrils from the following well characterized sources: rabbit psoas (fast), bovine masseter (slow) and bovine cardiac muscle. We confirmed the muscle composition using myosin-heavy–chain analyses, leading to the synthesis of CK-2017357 (molecular weight, 230.3; Supplementary Fig. 1a).

CK-2017357 selectively sensitizes fast skeletal muscle to calcium by binding to its troponin complex. We prepared nearly pure populations of fast, slow and cardiac myofibrils from the following well characterized sources: rabbit psoas (fast), bovine masseter (slow) and bovine cardiac muscle. The addition of CK-2017357 to fast skeletal myofibrils resulted in a leftward shift of the myosin ATPase relationship to calcium.

In many diseases, muscle weakness is the result of limited neural input and can lead to substantial disability and increased mortality. Peripheral motor neuropathies such as amyotrophic lateral sclerosis, spinal muscular atrophy and Charcot-Marie-Tooth disease cause motor neuron damage and death, which strains the ability of the surviving motor neurons to stimulate muscle effectively to generate force. In myasthenia gravis, weakness and fatigue result from the failure of signal transmission at the neuromuscular junction, which limits calcium release and force production. Therapy for myasthenia gravis consists of acetylcholinesterase inhibitors and immunosuppression, although weakness and fatigue are still common in affected individuals even after treatment. Therapeutic options for other neuropathies are limited or nonexistent.

We hypothesized that amplifying the response of the sarcomere, the fundamental contractile unit in skeletal muscle, to inadequate motor neuron input would improve muscle force generation and physical function in individuals with neuromuscular diseases. One way to amplify the sarcomere response is to increase the calcium sensitivity of the troponin-tropomyosin regulatory complex, which is the calcium sensor within the sarcomere that regulates the actin-myosin force-generating interaction. We identified a structural class of fast-skeletal–troponin activators using high-throughput screens of fast (type 2) and slow (type 1) skeletal myofibrils. We then optimized this compound class for potency, physicochemical properties and pharmacokinetics, leading to the synthesis of CK-2017357 (Supplementary Fig. 1a).

Skeletal muscle contraction occurs after motor neuron firing and the transmission of this impulse across the neuromuscular junction. The ensuing muscle action potential triggers the release of calcium from the sarcoplasmic reticulum. As the frequency of nerve firing increases, the amount of calcium released from the sarcoplasmic reticulum also increases, causing the force of muscle contraction to ramp up proportionally from a twitch to a maximal tetanic contraction. The relationship of the frequency of motor neuron firing to the corresponding muscle force is termed rate coding and is one of the means by which muscle strength is controlled. Under normal conditions, skeletal muscle operates in a range of forces between 10% and 65% of its maximum tetanic force.
concentration (pCa is the negative logarithm of the free calcium concentration) (Fig. 1b), with the pCa producing a half-maximal increase in ATPase (pCa_{50}) shifting from $5.61 \pm 0.01$ (mean \(\pm\) s.e.) before treatment (control) to $6.52 \pm 0.02$ after the addition of $5.0 \mu M$ CK-2017357. We characterized the potency of CK-2017357 by measuring the effect of increasing compound concentration on the myosin ATPase rate at a fixed calcium concentration (pCa = 6.0), resulting in a half maximal effective concentration (EC_{50}) for CK-2017357 treatment of $390 \pm 17 \mu M$ (mean \(\pm\) s.e.) (Fig. 1c). CK-2017357 had little or no effect in myofibrils from slow skeletal and cardiac muscle (Fig. 1c), showing its selectivity for fast skeletal muscle. Activation of myofibrils from bovine rectus abdominis muscle (EC_{50} = $770 \pm 100 \mu M$; Supplementary Fig. 1b), a muscle with mixed fiber types that contains, in part, fast skeletal muscle, confirmed its selectivity profile in muscle derived from the same species.

We leveraged this selectivity to determine the target of CK-2017357 using heterologous reconstituted versions of troponin-tropomyosin–regulated actin-myosin 15. CK-2017357 activated only those reconstructions containing the fast-skeletal–troponin complex (Fig. 1d). Isothermal titration calorimetry further confirmed a direct interaction of CK-2017357 with fast skeletal troponin ($K_{d} = 40 \pm 6 \mu M$ (mean \(\pm\) s.d.)). Addition of CK-2017357 to a purified fast-skeletal–troponin complex resulted in an exothermic reaction that fit well with a single-site–binding model. Consistent with its myofibril selectivity, CK-2017357 had a modest affinity for slow skeletal troponin ($K_{d} = 3,800 \pm 700 \mu M$) and no measurable affinity for cardiac troponin (Fig. 2a). This selectivity is not surprising, given the substantially different amino acid composition (~50%) of each corresponding troponin subunit isoform in slow skeletal and cardiac muscle.

The troponin complex contains three subunits, troponin C, troponin I and troponin T. Troponin C is a calcium sensor with four binding sites, two of which have high affinity ($K_{d} \approx 50 \mu M$) and two of which have low affinity ($K_{d} \approx 2 \mu M$). After calcium release from the sarcoplasmic reticulum, binding to the low-affinity sites results in tropomyosin movement and allows the actin-myosin interaction to proceed. As calcium is pumped back into the sarcoplasmic reticulum, calcium concentrations fall, the low-affinity sites release calcium and the muscle contraction ends. We measured calcium release from the low-affinity sites of fast skeletal troponin using a fluorescent calcium indicator in a stopped-flow apparatus. Addition of CK-2017357 (20 \mu M) slowed the first order rate constant for Ca^{2+} release from 14.7 s^{-1} (95% CI 14.5–14.8) to 4.0 s^{-1} (95% CI 3.9–4.1), which is consistent with CK-2017357 increasing the affinity of troponin for calcium (Fig. 2b). Similar experiments with isolated fast skeletal troponin C did not show either a change in the calcium release rate (Fig. 2c) or a binding interaction (Fig. 2d), suggesting that the CK-2017357 binding site lies at an interface between two or more troponin subunits.

We sought to understand how an increase in troponin calcium affinity would translate into changes in muscle force. We prepared chemically ‘skinned’ human muscle fibers with the plasma membranes rendered freely permeable to Ca^{2+} from muscle biopsies of the vastus lateralis, which is a mixed fast (type 2a, 2x myosins) and slow (type 1 myosins) muscle. These fibers contract when exogenous calcium is added to the muscle fiber. Treatment of fast fibers with CK-2017357 shifted the plot of the force-calcium relationship.

Figure 1 CK-2017357 is a selective calcium sensitizer of the fast-skeletal–troponin complex. (a) The chemical structure of CK-2017357. (b) Calcium dependence of the fast skeletal myofibril ATPase at two concentrations of CK-2017357 compared to vehicle control. (c) The dose response of CK-2017357–treated fast skeletal, slow skeletal and cardiac myofibrils at a pCa of 6.0. (d) Activation of heterologous, reconstituted thin filaments (with the source of the regulatory complex isoform being cardiac (C) or fast skeletal (FS) muscle) by CK-2017357 using cardiac myosin ATPase as a probe for thin-filament activation at a pCa of 6.75, which is the approximate pCa that produces a one-fourth–maximal force (pCa_{25}) for all systems. Values are mean \(\pm\) s.d.

Figure 2 CK-2017357 binds to the skeletal-troponin complex and slows calcium release. (a) Isothermal titration calorimetry of CK-2017357 that was added into purified cardiac, slow skeletal and fast skeletal intact troponin complexes (bottom). The three top plots are from representative experiments for the heats of addition to each isoform during the CK-2017357 titration. (b,c) Fluorescence intensity of the calcium chelator quin-2 after rapid mixing with intact fast skeletal troponin (b) or recombinant rabbit fast skeletal muscle troponin C (c) in the presence or absence (control) of CK-2017357. (d) The heats of addition (above) and isothermal titration calorimetry (below) of CK-2017357 added to recombinant rabbit fast skeletal troponin C (TnC).
Figure 3 CK-2017357 shifts the plot of the force-calcium relationship in fast skeletal muscle leftwards and amplifies the response of muscle to nervous input. (a) The force-calcium relationship in human skinning fast (left) and slow (right) skeletal muscle fibers at three concentrations of CK-2017357. Force measurements (for each condition, n = 10) are shown as a fraction of the maximum force. (b) The force-calcium relationship of single skinned fibers from rabbit fast skeletal psoas muscle fibers (top, n = 12), rat slow skeletal soleus muscle (middle, n = 13) and rat cardiac muscle fibers (bottom, n = 10) treated with CK-2017357. (c) The time-frequency relationship of rat EDL muscle in situ before and after treatment with CK-2017357 (n = 6). Values are means ± s.e.m.

Figure 4 CK-2017357 improves muscle and physical function in a model of neuromuscular disease. (a) Rat forelimb grip strength in PT-EAMG rats with time after administration of anti-nAChR antibody or phosphate buffered saline (control). n = 10 for each group. BW, body weight; mAb, monoclonal antibodies. (b) The force-frequency relationship of rat EDL muscle in situ 72–96 h after intra-peritoneal injection of anti-nAChR antibody (PT-EAMG, n = 6) or phosphate-buffered saline (control, n = 4). (c) In situ rat EDL peak muscle force with time after infusion of CK-2017357 (n = 6) or vehicle (n = 4) in PT-EAMG rats. (d) In situ rat EDL muscle force with time for a single representative contraction before and after administration of CK-2017357 in PT-EAMG rats. (e) Decline in muscle force (sag) under the same stimulation protocol in control rats (n = 4), PT-EAMG rats (n = 6) or PT-EAMG rats after infusion of CK-2017357 (n = 6). (f) Forelimb grip strength in PT-EAMG rats 60 min after a single oral dose of CK-2017357 (n = 20–22 in each dose group). *P < 0.001 compared to vehicle treatment, aP < 0.01 compared to the 5 and 10 mg per kg of body weight doses using an analysis of covariance. (g) Rat forelimb grip strength change after oral administration of CK-2017357 (n = 14) or vehicle (n = 14) in healthy rats. For all graphs, means ± s.e.m. are plotted.

leftwards without increasing the maximum recorded force or the shape of the curve; in contrast, slow fibers were approximately tenfold less responsive to CK-2017357 than the fast fibers (Fig. 3a).

Skinned skeletal muscle fibers from rabbit (fast fibers) and rat (slow fibers) showed similar fiber-type selectivity to the human skeletal muscle fibers and, notably, skinned cardiac muscle fibers obtained from rat heart were unresponsive to CK-2017357 (60 µM) (Fig. 3b). These experiments confirmed the selectivity of CK-2107357 for fast skeletal muscle in an intact muscle system.

Given its effects on calcium sensitivity, the addition of CK-2017357 increases in muscle force (Supplementary Fig. 2). Examination of the force-frequency relationship in this muscle revealed a substantial increase in muscle force at sub-maximal stimulation rates as a result of treatment without increasing the maximum tension at the maximal tetanic stimulation rates (Fig. 3c). Tension in the absence of nerve stimulation did not change after treatment (data not shown). Thus, the change in the force-frequency relationship seen here as a result of treatment was consistent with the effect of CK-2017357 on the force-calcium relationship in human and rabbit fast skinned muscle fibers.

The leftward shift of the force-frequency relationship of rat fast skeletal muscle in situ led us to test whether CK-2017357 could increase muscle force in a model of neuromuscular disease. Passive transfer experimental autoimmune myasthenia gravis

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(PT-EAMG) is a rat model of myasthenia gravis in which treatment with an inhibitory monoclonal antibody that binds to the α1, α3 and β5 isoforms of the nicotinic acetylcholine receptor (nAChR) leads to muscle weakness and fatigue. Injection with this antibody (anti-nAChR antibody) led to a substantial decline in grip strength (~50%) in PT-EAMG rats, reaching a plateau at 72 h (Fig. 4a) that remained stable at 96 h after administration. Underlying this weakness in grip strength was a decrease in muscle force caused by anti-nAChR antibody treatment when measured in situ across a wide range of nerve-stimulation frequencies (Fig. 4b). In an in situ preparation of the EDL in these PT-EAMG rats 72 to 96 h after anti-nAChR antibody treatment, administration of CK-2017357 rapidly increased muscle force (Fig. 4c) and eliminated the decline in force produced by prolonged stimulation, the so-called use-dependent fatigue or sag (Fig. 4d,e).

We next tested whether these increases in the performance of individual, intact muscles could translate to enhanced physical performance in the same PT-EAMG model. After randomization of the PT-EAMG rats into two groups, we orally dosed the rats with either CK-2017357 or vehicle in a blinded fashion at 72 h after injection of anti-nAChR antibody, which we followed with a cross-over to the opposite treatment at 96 h after injection. We performed grip-strength assessments 60 min after CK-2017357 administration. CK-2017357 treatment increased the grip strength of treated rats by up to 0.97 kg per kg of body weight relative to the increase seen in rats treated with vehicle 60 min after dosing, representing a more than 50% increase in grip strength in the treated rats relative to baseline (Fig. 4f). Of note, control rats (saline injection instead of anti-nAChR antibody injection) randomized to CK-2017357 or vehicle treatment 72 h after their injection did not show a change in grip strength 60 min after treatment with CK-2017357 compared to baseline and to the vehicle-treated group (Fig. 4g), suggesting that the increases in muscle function caused by CK-2017357 in this setting required prior weakness produced by neuromuscular blockade.

CK-2017357 is a selective sensitizer of the fast-skeletal–troponin complex and a direct activator of skeletal muscle function. CK-2017357 may be a therapeutic option for a range of serious neuromuscular disorders by increasing muscle strength and reducing fatigability. Evidence also points to a reduced efficiency of motoneuron excitation-contraction coupling in the sarcopenia of old age, suggesting a potentially broader applicability of this mechanism of action. Direct activators of the skeletal sarcomere, such as the fast-skeletal–troponin activator CK-2017357, may therefore hold promise in an array of conditions marked by muscle weakness.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

All authors contributed extensively to the work presented in this paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

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ONLINE METHODS

Materials. We used a previously described method for our glycerol SDS-PAGE analysis of myosin isoforms. We prepared rabbit fast skeletal myofibrils from psoas muscle, slow skeletal myofibrils from bovine masseter muscle and cardiac myofibrils from bovine heart muscle. Myofibrils were also prepared from bovine rectus abdominis muscle, a muscle predominantly comprised of fast fibers. We purified myosin (as the S1 fragment produced by limited proteolytic treatment with chymotrypsin), actin, troponin and tropomyosin from rabbit psoas muscle for skeletal isoforms and from bovine heart for cardiac isoforms. We reconstituted versions of troponin-tropomyosin–regulated actin-myosin according to published methods. For the in vitro skinned-fiber studies, we prepared muscle as previously described. Human skeletal muscle tissue was obtained by needle biopsy from the vastus lateralis muscles of volunteer subjects after obtaining signed informed consent under a protocol reviewed and approved by the institutional review boards of the University of Michigan Medical School. Cytokinetics Institutional Animal Care and Use Committee reviewed and approved the protocols related to the rodent models. Fluorescent reagents were purchased from Molecular Probes. Cytokinetics synthesized the CK-2017357, and the stock solutions for the in vitro testing were prepared in DMSO. The final DMSO concentration in the test solutions was 1–2%.

ATPase assays. We measured the steady state ATPase activity at 25 °C using a pyruvate kinase and lactate dehydrogenase–coupled enzyme system and a SpectraMax plate reader ( Molecular Devices) to monitor the change in absorbance as a function of time. The buffer used was 12 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 2 mM MgCl₂ and 1 mM dithiothreitol at pH 6.8, with 60 mM KCl being added for fast and slow skeletal myofibrils.

Isothermal titration calorimetry. We did isothermal titration calorimetry experiments in a Micro-Cal VP-ITC MicroCalorimeter (General Electric) using 50 µM rabbit fast-skeletal–troponin complex or purified recombinant rabbit fast troponin C in PIPES buffer (12 mM K-PIPES pH 6.8, 100 mM KCl, 250 µM CaCl₂, 5 mM β-mercaptoethanol and 3% DMSO). The reference chamber contained PIPES buffer and 3% DMSO. The ligand solution injected into the sample chamber contained CK-2017357 (300 µM). Ligand injections were made every 300 s (10 µl). To correct for the heats of dilutions of CK-2017357, we subtracted the stable heat signal from the injections near the end of the experiment from all values and used the instrument software package for data collection and analysis using a single-binding–site model.

Measurement of calcium release from troponin. We measured the calcium release from recombinant troponin C or the troponin complex using the fluorescent calcium chelator quin-2 (ref. 32) in an SF-61DX stopped-flow fluorimeter (TgK Scientific), with excitation provided by a monochromator (337 nm, with a 10-nm slit width) and emission measured through a glass filter passing wavelengths of 495 nm or greater. Calcium standard curves prepared in the presence of the equivalent concentrations of DMSO and CK-2017357 allowed for the translation of the fluorescence intensities into calcium concentrations. The final reaction conditions were as follows: 10 µM troponin C or troponin complex (pre-incubated with equimolar supplemental CaCl₂), 1% DMSO (± 20 µM CK-2017357), 75 µM quin-2, 12 mM K-PIPES pH 6.8, 2 mM MgCl₂ and 1 mM dithiothreitol at 25 °C.

Muscle force measurements. We performed skinned-fiber studies as previously described. After attaching human fibers with 10-0 monofilament nylon, we measured muscle force with a Model 403A force transducer and Model 322C servomotor ( Aurora Scientific) at a sarcomere length of ~2.7 µm.

Statistical analyses. Based on the crossover design of the grip-strength measurement data, we performed statistical analyses of change in grip strength from baseline to active treatment and vehicle using an analysis of covariance procedure, with the rats included in the model as a random effect, baseline grip strength included as a covariate and treatment, sequence of treatment and treatment occasion (that is, the first or second treatment) included as fixed factors. Unless otherwise noted, values in the text are reported as mean ± s.e.m.

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