

THE FAST SKELETAL TROPONIN ACTIVATOR, CK-2017357, INCREASES SKELETAL MUSCLE FORCE *IN VITRO* AND *IN SITU*

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INTRODUCTION

Direct modulation of muscle contractility at the level of the contractile apparatus is a therapeutic approach with applicability to several diseases. Previous discovery efforts directed at cardiac muscle resulted in the identification of CK-1827452, a small molecule direct activator of cardiac myosin that increases cardiac contractility and is currently being studied in Phase II clinical trials in patients with systolic heart failure. Similarly, a small molecule activator of the skeletal sarcomere may have equal utility in increasing muscle function in patient groups where skeletal muscle weakness is a feature.

CK-2017357 is a fast skeletal troponin activator that was discovered as part of a screening and chemical optimization process using detergent treated skeletal muscle myofibrils from rabbit muscle. In biochemical assays, it sensitizes the fast skeletal myofibril ATPase activity to calcium, shifting the pCa relationship to the left without affecting enzymatic activity at low and high calcium concentrations.

The objective of this study was to evaluate whether CK-2017357 changes force development in native skeletal muscle preparations *in vitro*, using skinned and living skeletal muscle fibers, and *in situ*, where nerve and blood supply are left intact. We also determined the activity of CK-2017357 in skeletal muscle fibers isolated from fast (type IIa, IIb, IIx/d) versus slow (type I) or cardiac muscle to assess tissue selectivity.

METHODS

Isometric skinned fiber analysis: Muscle fibers for skinned fiber studies were prepared using a protocol based on Lynch and Faulker, 1998. Single muscle fibers were dissected in rigor buffer at 4°C (20 mM MOPS, 5 mM MgCl₂, 120 mM potassium acetate, 1 mM EGTA, pH 7.0) and attached to a 400A force transducer (Aurora Scientific, Ontario, Canada) with 2-4 µl of a 5% solution of methylcellulose in acetone. Fibers were incubated at 10°C in relax buffer (20 mM MOPS, 5.5 mM MgCl₂, 132 mM potassium acetate, 4.4 mM ATP, 22 mM creatine phosphate, 1 mg/mL creatine kinase, 1 mM DTT, 44 ppm antifoam, pH 7.0) and baseline tension adjusted. Tension was generated by incubating fibers in relax buffer supplemented with 1 mM EGTA and 10 nM to 100 µM free calcium ions (labeled as pCa 8 to pCa 4, added as different volumes of a 15 mM solution of CaCl₂ and calculated using the web resource <http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm>). Compound was added to these buffers from a DMSO stock (final DMSO concentration 1%).

***In vitro* muscle analysis:** Adult male Sprague-Dawley rats were euthanized and a small branch of the flexor digitorum brevis (FDB) was dissected from the foot in oxygenated Krebs solution at 4°C (1 mM NaH₂PO₄, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 137 mM NaCl, 11 mM glucose and 1 mM NaHCO₃). Muscles were attached with silk thread to the fixed lever arm and force transducer of an 801A *in vitro* analysis system (Aurora Scientific, Ontario, Canada) and perfused with Krebs solution at 20°C. After length adjustment, muscles were stimulated via field electrodes with 350 ms trains (5, 10, 20, 30, 50, 80, 100 Hz) over a 2 minute period. This was repeated every 10 minutes. For compound treatment, muscles were perfused with DMSO (0.1%) or CK-2017357 (1-10 µM). At the end of each assay, the length and weight of the muscle was recorded, and measured force normalized to the cross sectional area of the muscle (N/cm², described in Segal and Faulkner, 1985).

***In situ* muscle analysis:** *In situ* studies were based on experimental procedures described in Brooks et al., 1990. Rats were placed under anesthesia using isoflurane and the distal end of the extensor digitorum longus (EDL) muscle and its associated tendon were isolated. The knee was immobilized with a clamp and the tendon cut and tied to the arm of a force transducer (806C, Aurora Scientific) using silk suture. The muscle was stimulated directly via the peroneal nerve at the upper thigh with a pair of stainless steel hook electrodes. Muscle length was adjusted to produce maximum isometric force (Lo) and then stimulated every 2 minutes with a 30 Hz train (1 ms stimuli, 350 ms duration) for the course of the experiment. CK-2017357 was administered as a 2 minute bolus in increments up to a total of 10 mg/kg via the femoral artery as a solution (50% PEG300/10% EtOH/40% cavitron). For analysis of the force/frequency relationship, muscles were stimulated at 10 Hz to 200 Hz before and after treatment with 10 mg/kg CK-2017357 over a 2 minute period. At the end of each assay, the length and weight of the muscle was recorded, and measured force normalized to the cross sectional area of the muscle (N/cm²).

CK-2017357 Selectively Activates Fast Skeletal Muscle

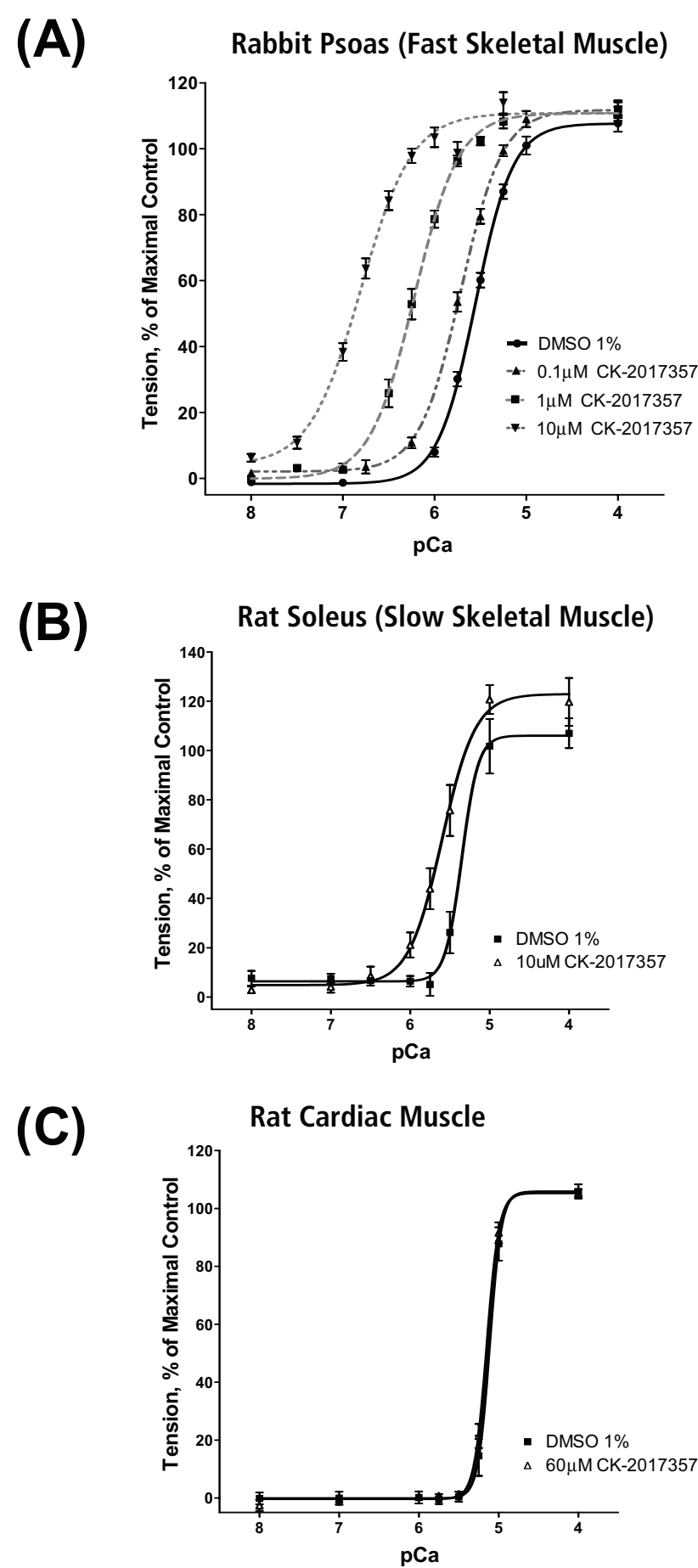


Figure 1. Effect of CK-2017357 on skinned skeletal fibers from (A) rabbit psoas muscle (fast skeletal muscle, n=8), (B) rat soleus muscle (slow skeletal muscle), (C) rat cardiac muscle. Single skinned fibers were attached to a model 400A force transducer (Aurora Scientific) at 10°C and force measured after incubation with increasing concentration of buffered calcium and the indicated concentration of CK-2017357 (force is plotted as a percent of maximal contraction measured at pCa 4).

CK-2017357 Activates Human Fast Skeletal Muscle Fibers

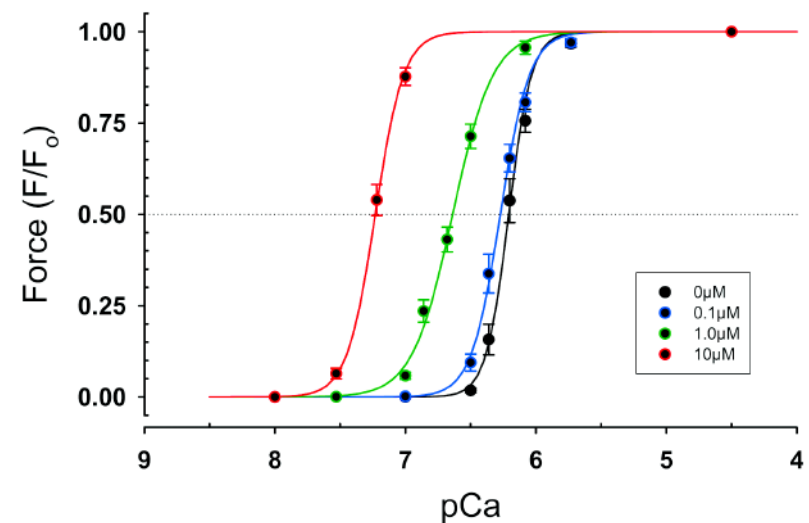


Figure 2. CK-2017357 Increases Force Development in Permeabilized Human Fast Skeletal Muscle Fibers. Single fibers were isolated from muscle bundles and were secured to a force transducer and servomotor (both Aurora Scientific, Inc.). Force was measured by immersing fibers in a series of solutions, each having a different, well-buffered [Ca²⁺]. Fast fibers (IIa and IIx/d) were classified based on their isovelocity shortening properties. Force measurements are displayed as a fraction of maximal force (F₀) measured at pCa 4 before compound treatment (n=10).

RESULTS

CK-2017357 Increases Force Development in Living Muscle

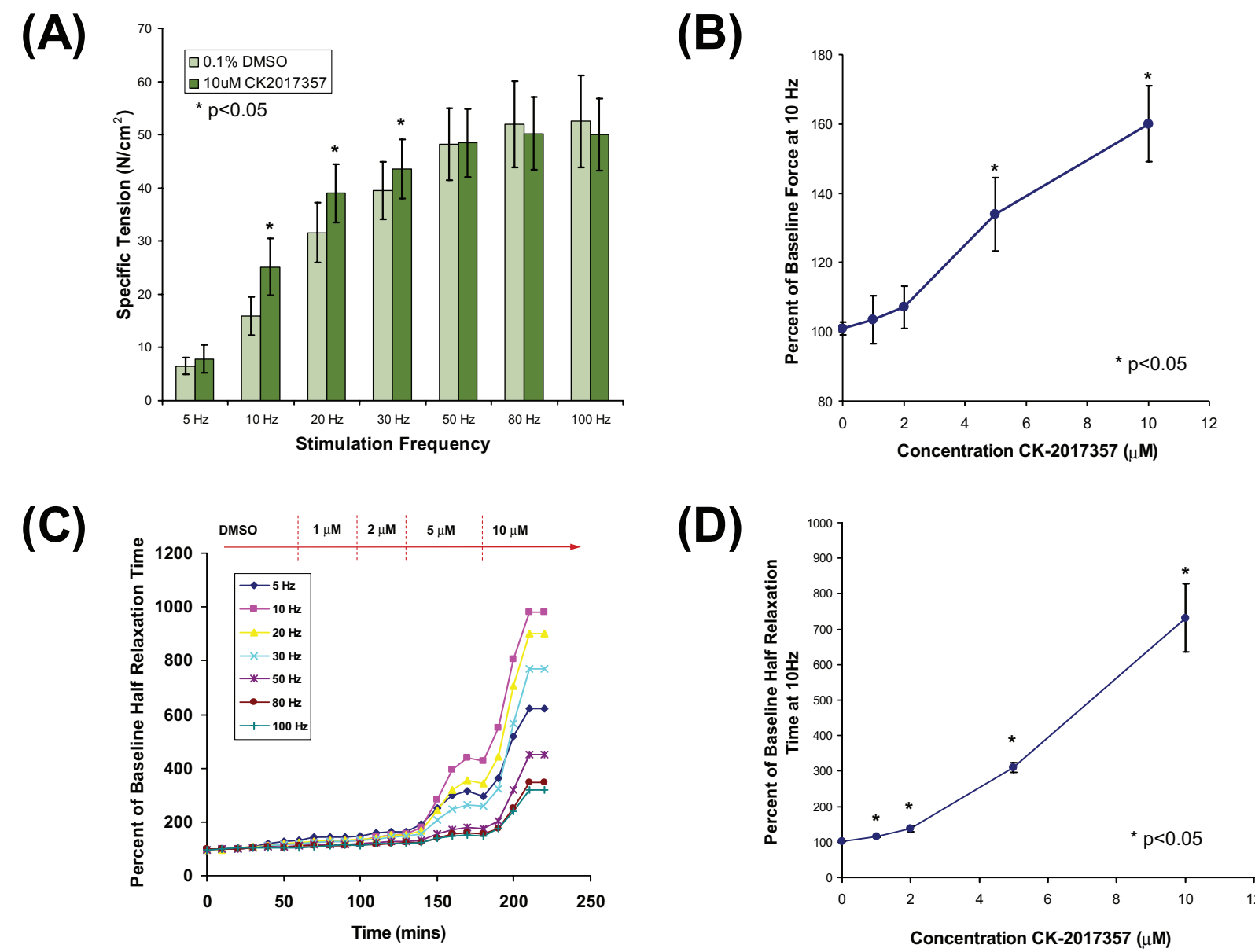


Figure 3. CK-2017357 Increases Force Development in Rat Flexor Digitorum Brevis (FDB) Muscle *in vitro*. FDB muscles (approx 85% fast fiber composition) were incubated at 20°C in Krebs buffer. (A) Effect of 10 µM of CK-2017357 on the force/frequency relationship in FDB muscles (specific tension (+/- sd); * p<0.05 vs pretreatment; n=6). (B) Graph showing the average change in force (+/- sd) at 10 Hz vs concentration of CK-2017357 (n=7, * p<0.05 vs DMSO). (C) Result from a single experiment showing the change in half relaxation time of the muscle with time at seven different stimulation frequencies with increasing concentrations of CK-2017357. (D) Graph showing the average change in relaxation time (error +/- sd) at 10 Hz vs concentration of CK-2017357 (n=7, * p<0.05 vs DMSO).

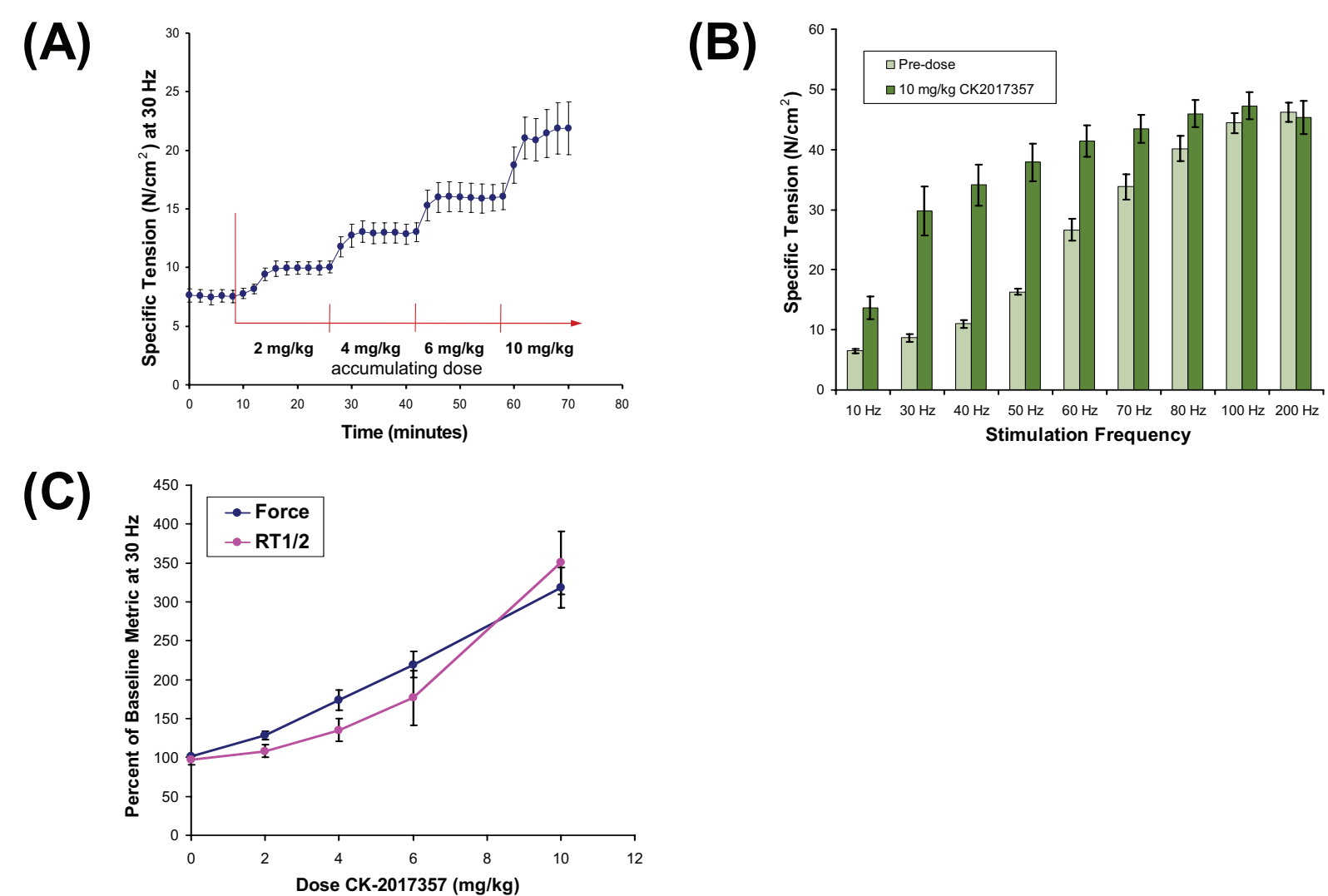
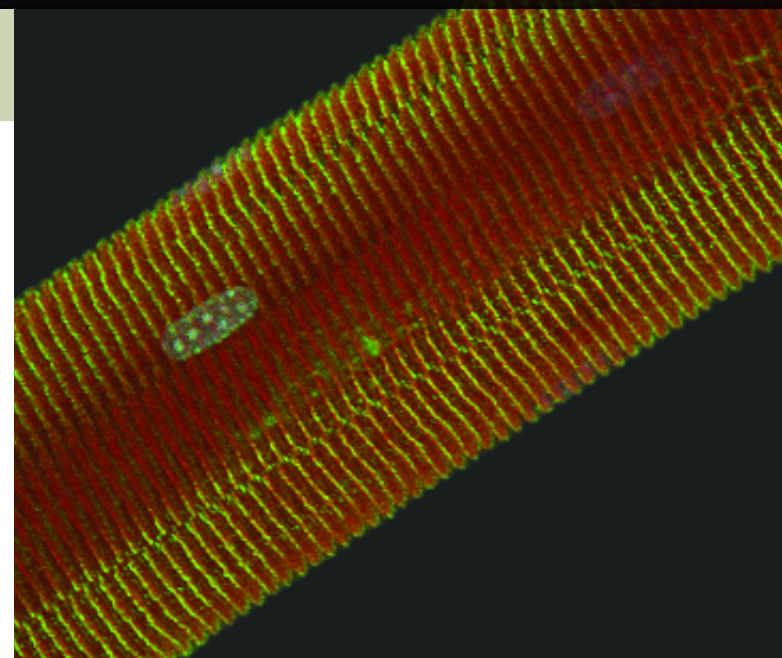


Figure 4. CK-2017357 Increases Force Development in Rat Extensor Digitorum Longus (EDL) Muscle *in situ*. (A) The EDL muscle (approx 90% fast fiber composition) was stimulated every 2 minutes at 30 Hz via the peroneal nerve. CK-2017357 was then administered as a 2 minute intra-arterial bolus in four cumulative doses up to 10 mg/kg (n=5, error bars +/- sem). Force is plotted as N/cm², normalized to the weight and cross-sectional area of the muscle. (B) Force/Frequency plot pre- and post-treatment with 10 mg/kg of CK-2017357 (n=5, error +/- sd). (C) CK-2017357 increases relaxation time in proportion to force *in situ*. Plotted is the percent increase in force and half relaxation time (RT1/2) at 30 Hz following escalating doses of CK-2017357 (n=7, error +/- sem).



CONCLUSIONS

1. The skeletal troponin activator CK-2017357 increases sub-maximal force development in fast skeletal rabbit and human muscle *in vitro*.
2. In living rat FDB preparations, increases in force are coupled to frequency-independent increases in relaxation time.
3. Skinned slow skeletal muscle fibers are approximately tenfold less responsive to CK-2017357 than skinned fast skeletal muscle fibers, confirming the compound selectivity for fast skeletal muscle fibers. CK-2017357 does not activate cardiac muscle.
4. CK-2017357 increases sub-maximal force development in the EDL muscle of rats after arterial administration of compound. In contrast to *in vitro* results, the increases in relaxation time are attenuated and directly proportional to increases in force.

These data support the proposed mechanism of action of the fast skeletal troponin activator, CK-2017357. In skinned muscle fibers, CK-2017357 increases the sensitivity of skeletal muscle to calcium and in living muscle to the frequency of stimulation, each of which results in an increase in muscle force development at sub-maximal muscle activation. These findings may translate into functional improvements in skeletal muscle performance in conditions marked by muscle weakness by improving the extent of muscle fiber recruitment during physical activity.

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