The Fast Skeletal Troponin Activator, CK-2017357, Reduces Muscle Fatigue in an *in situ* Model of Vascular Insufficiency

Aaron C Hinken, Lena Driscoll, Ken Lee, Jim J Hartman, David Marquez, Richard Hansen, Alex Muci, Bradley Morgan, David J Morgans Jr, Alan J Russell, Fady Malik

Cytokinetics, Inc., South San Francisco, CA, USA.

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 *omecamtiv mecarbil*, 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 the effects of CK-2017357 on the time to fatigue in native skeletal muscle preparations *in vitro*, and in skeletal muscle, *in situ*, where blood supply and nervous input to the muscle is left intact. In addition, the time to fatigue *in situ* was examined after the blood supply was limited by occlusion of the femoral artery

Results

CK-2017357 increases tension in muscle fibers and whole muscle.

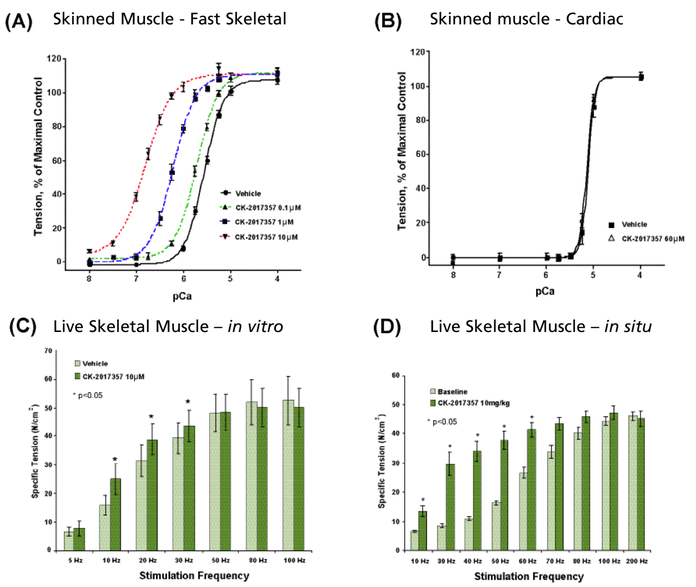


Figure 1. CK-2017357 increases sub-maximal tension development in fast skeletal muscle from muscle fiber to whole muscle. (A) CK-2017357 increases Ca2+-sensitivity of isolated, skinned rabbit psoas muscle fibers. Single skinned fibers were attached to a model 400A force transducer (Aurora Scientific) at 10°C and force measured after incubation with varied concentrations of buffered calcium and the indicated concentration of CK-2017357 (force is plotted as a percent of maximal contraction measured at pCa 4 without compound; mean force +/- S.D.). **(B)** CK-2017357 does not affect single skinned cardiac fibers (using methods as indicated for fast skeletal muscle), indicating fast skeletal specificity. **(C)** CK-2017357 increases sub-maximal force development of rat flexor digitorum brevis (FDB) muscle *in vitro*. FDB muscles (approx 85% fast fiber composition) are incubated at 20°C in Krebs buffer; the effect of 10 µM CK-2017357 on the force-frequency relationship is shown (mean specific tension +/- S.D.; * p<0.05 vs baseline; n=6). **(D)** CK-2017357 increases force in rat extensor digitorum longus (EDL) muscle *in situ*. The EDL muscle (approx 90% fast fiber composition) was stimulated every 2 minutes at 30 Hz via the peroneal nerve until stable, followed by determination of the baseline force-frequency relationship. CK-2017357 was then administered as a 2 minute intra-arterial infusion. The force-frequency plot shows the baseline and treatment effects of 10 mg/kg CK-2017357 (n=5, mean specific tension +/- S.D.). Force is plotted as N/cm², normalized to the weight and cross-sectional area of the muscle.

Results (contd.)

CK-2017357 decreases fatigue *in vitro* and *in situ*.

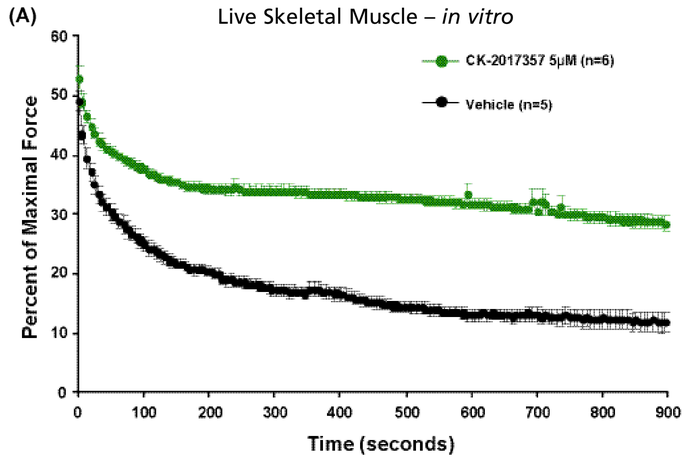


Figure 2. CK-2017357 decreases fatigue in skeletal muscle preparations *in vitro* and *in situ* during normal conditions. Fatigue protocol is intended to be representative of moderate exercise fatigue, not maximal fatigue. Graphs show the percent of maximal force over time (+/- S.E.M.). **(A)** *In vitro:* FDB muscles were pre-incubated for 30 min at 4°C in Krebs buffer at resting tension with DMSO (0.1%) or CK-2017357 (10 µM). Incubation temperature was then raised to 30°C and a force-frequency relationship established (350 ms trains at 5, 10, 20, 30, 50, 80, 100 Hz). Stimulation frequency was adjusted to achieve a force of 50% of maximal (FMax50) and muscles were stimulated at this frequency every 6 seconds for 15 minutes. **(B)** *In situ:* The EDL is stimulated with 350ms trains every 3 seconds for ten minutes at a frequency producing an initial force equal to 50% of maximal (FMax50) as determined by the force-frequency relationship of each animal. Compound or vehicle is delivered via duodenal cannula.

Measuring rat EDL function during vascular insufficiency.

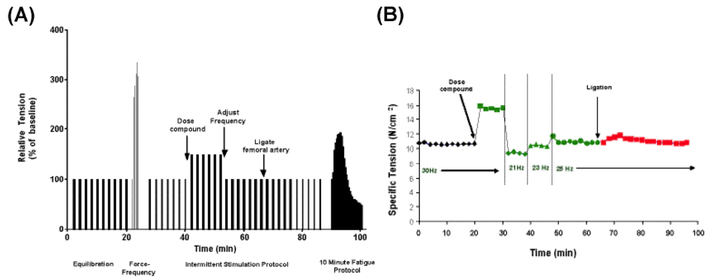


Figure 3. Experimental assessment of changes in rat EDL function *in situ* with a surgical model of vascular insufficiency. (A) The EDL muscle is isolated, attached to a force transducer, and stimulated at 30 Hz every two minutes until equilibrium is achieved. The force-frequency relationship is then determined, as a quality control measure. Next a baseline is established, vehicle or compound is administered, stimulation frequency is adjusted as necessary to match pre-administration force production, and the femoral artery is ligated. Finally, a 10-minute fatigue protocol is conducted. **(B)** A representative experiment showing force over the duration of the pre-fatigue experimental protocol, including response to compound administration and subsequent titration of stimulation frequency to match pre-administration force level.

CK-2017357 increases the time to fatigue after femoral artery ligation.

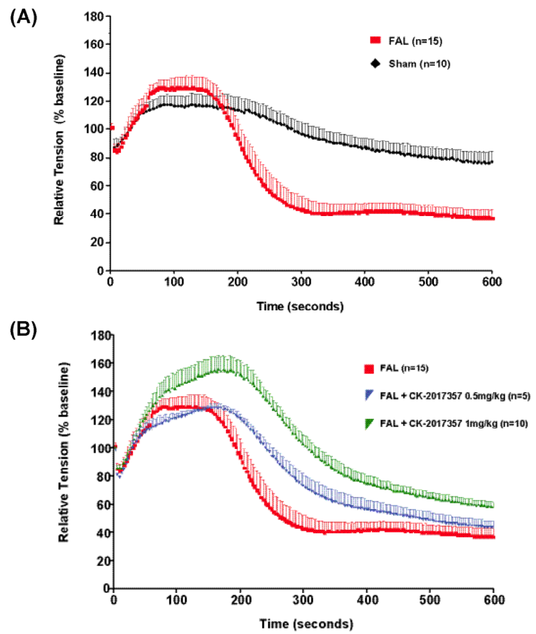
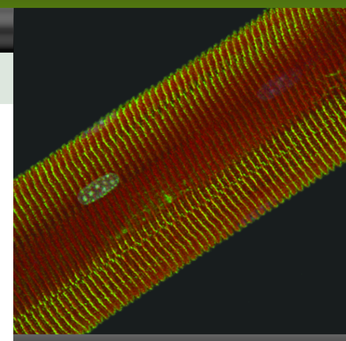


Figure 4. CK-2017357 increases the time to fatigue in skeletal muscle after femoral artery ligation (FAL). The EDL is stimulated with 350 msec trains every 3 seconds for ten minutes at a frequency that produces the same force as baseline. Vehicle animals are stimulated at 30Hz for entire experiment; CK-2017357 treated rats are stimulated at a frequency that matches the force produced at 30 Hz prior to compound administration. **(A)** Time to fatigue, indexed as time to 50% initial tension, and total tension generating capacity (AUC of tension) were significantly reduced in vehicle treated animals after femoral artery ligation, as compared to sham operated animals. **(B)** Treatment with CK-2017357 (average stimulation frequency of 29 and 26Hz, for 0.5 and 1mg/kg, respectively) produces a dose-dependent increase time to fatigue and tension generating capacity in FAL animals as compared to vehicle-treated FAL animals.



Conclusions

1. The fast skeletal troponin activator, CK-2017357, increases:
   a. the calcium-sensitivity of force production in skinned fast skeletal muscle
   b. sub-maximal force development in isolated fast skeletal muscle *in vitro*
   c. sub-maximal force development in the EDL muscle of rats *in situ*
   d. the time to overall fatigue after repetitive stimulation
2. In addition, the fast skeletal troponin activator, CK-2017357 ameliorates the increase in fatigability induced by vascular insufficiency *in situ* in a rodent model of claudication.

These data are consistent with the 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. In addition, CK-2017357 reduces isometric muscle fatigue *in vitro* and *in situ* under physiological conditions. Moreover, CK-2017357 increases the time to muscle fatigue *in situ* when blood supply is restricted.

These findings suggest that sensitization of the troponin complex to calcium, as mediated by the fast skeletal troponin activator, CK-2017357, has the potential to ameliorate muscle dysfunction induced by peripheral vascular insufficiency, such as exists in claudication.

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