

FAST SKELETAL TROPONIN ACTIVATOR, CK-1909178, REDUCES MUSCLE FATIGUE IN A MODEL OF PERIPHERAL ARTERY DISEASE *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 *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-1909178 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, they sensitize 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 fast skeletal troponin activator CK-1909178 on fatigue in native skeletal muscle preparations *in vitro*, using intact skeletal muscle under normal and hypoxic conditions, and *in situ*, where nervous input is left intact but the blood supply is limited by occlusion of the femoral artery.

MATERIALS AND 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 403A 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/webmaxc5.htm>). Compound was added to these buffers from a DMSO stock (final DMSO concentration 1%).

***In vitro* muscle analysis:** A small branch of the flexor digitorum brevis (FDB) was dissected from the foot of 1 adult male Sprague-Dawley rat 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 incubated in 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, 1 ms stimuli) over a 2-minute period. Prior to fatigue assays muscles were perfused with DMSO (0.1%) or CK compound for 60 minutes at 4°C. Incubating 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, 1 ms stimuli). Stimulation frequency was adjusted to achieve a force of 50% of maximal (FMax50) and muscles were stimulated at this frequency every 3 seconds for 5 minutes. Hypoxia was induced by removal of normal oxygenated buffer (95% oxygen and 5% carbon dioxide) for approximately 1 hour. At the end of each assay, the length and weight of each muscle were recorded, and measured force was 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 by 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 ipsilateral femoral artery branch was isolated and multiple loose knots of silk suture placed around the vessel distal to the internal iliac branch. 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. For analysis of the force/frequency relationship, muscles were stimulated at 10 Hz to 200 Hz before treatment over a 2-minute period. CK-1909178 was administered as a 2-minute bolus via the jugular vein as a solution (50% PEG300/10% EtOH/40% cavitron), producing an increase in force. The frequency of the train stimulation was titrated to match pre-treatment force levels. Constriction of the sutures around the femoral artery restricted blood flow to the lower leg, as described previously (Orito et al, 2004 and Okyayuz-Baklout, 1989). This procedure has been shown to decrease blood flow to the muscles of interest acutely by ~80% with negligible changes in basal tension (Okyayuz-Baklout, 1989). After force stabilized, the EDL was fatigued at 30Hz or the titrated frequency with stimulations every 3 seconds (1 ms stimuli, 350 ms duration) for ten minutes. At the end of each assay the length and weight of the muscle was recorded. The measured force was normalized to the cross-sectional area of the muscle (N/cm²).

CK-1909178 Sensitizes Fast Skeletal Muscle

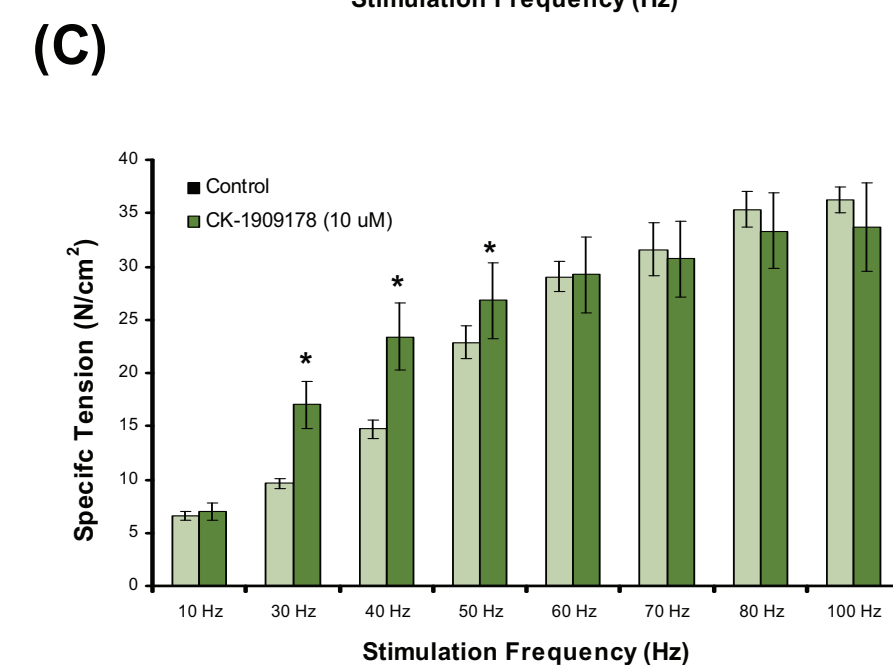
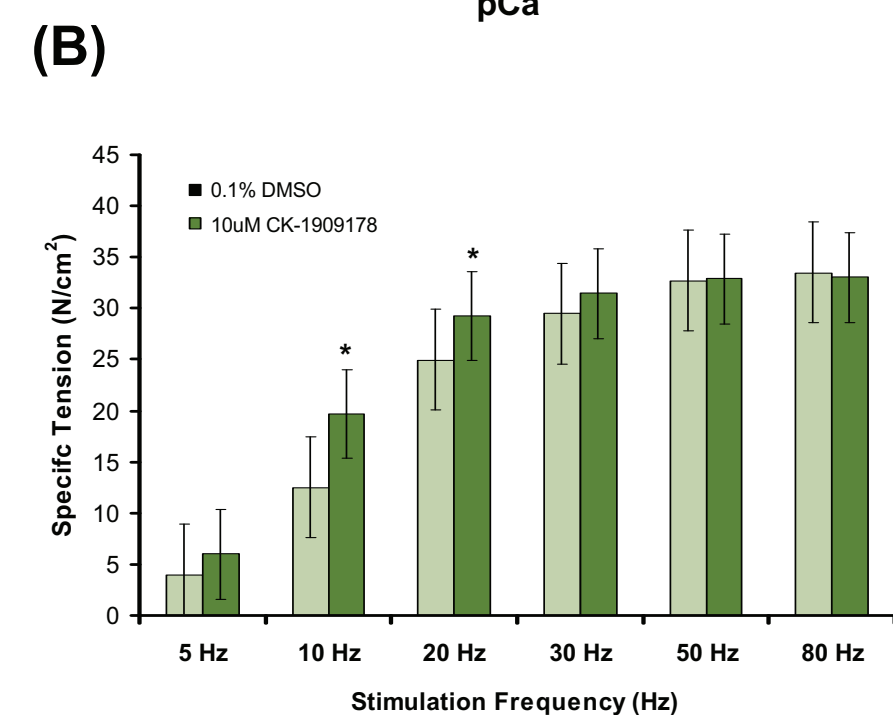
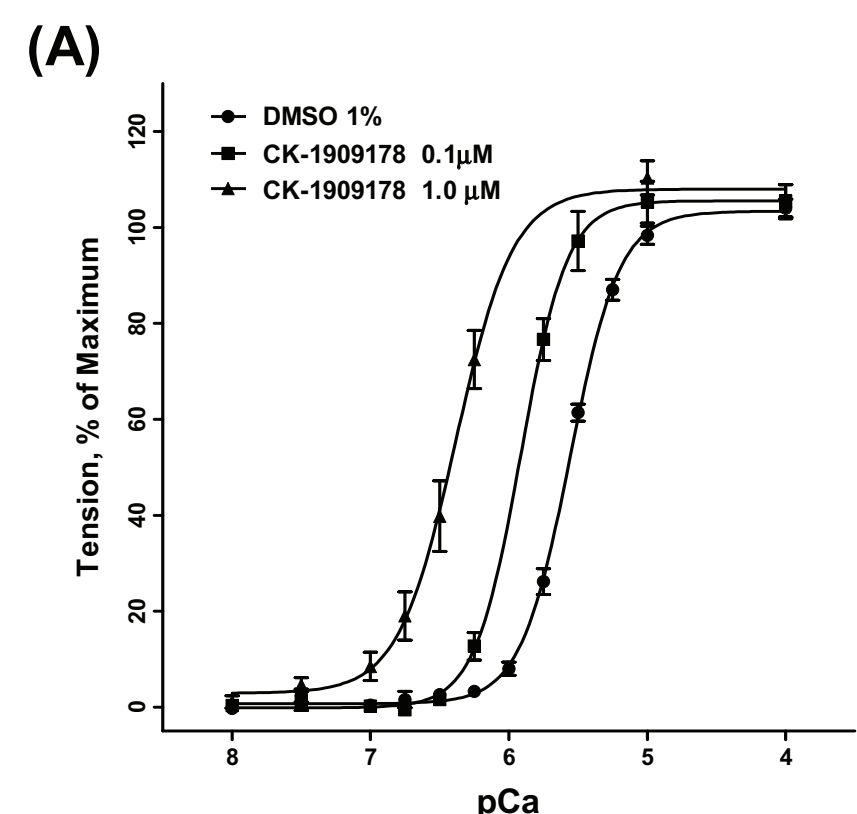


Figure 1:

Fast troponin activator CK-1909178 increases sub-maximal tension responsiveness of fast skeletal muscle from fiber to whole muscle level.

(A) CK-1909178 increases Ca-sensitivity of isolated, skinned rabbit psoas muscle fibers. Single skinned fibers were attached to a model 403A force transducer (Aurora Scientific) at 10°C and force measured after incubation with varied concentration of buffered calcium and the indicated concentration of CK-1909178 (force is plotted as a percent of maximal contraction measured at pCa 4).

(B) CK-1909178 increases *in vitro* sub-maximal force development of rat Flexor Digitorum Brevis (FDB) muscle. FDB muscles (approx 85% fast fiber composition) incubated at 20°C in Krebs buffer, with effect of 10 μM CK-1909178 on the force/frequency relationship shown (specific tension +/- S.D.; *p<0.05 vs. pretreatment; n=6).

(C) CK-1909178 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 baseline force-frequency relationship determination. CK-1909178 was then administered as a 2-minute intra-arterial bolus with Force-Frequency plot showing pre- and post-treatment effects of 10 mg/kg CK-1909178 (n=5, error +/- sd). Force is plotted as N/cm², normalized to the weight and cross-sectional area of the muscle.

RESULTS

CK-1909178 Decreases Fatigue *in vitro*

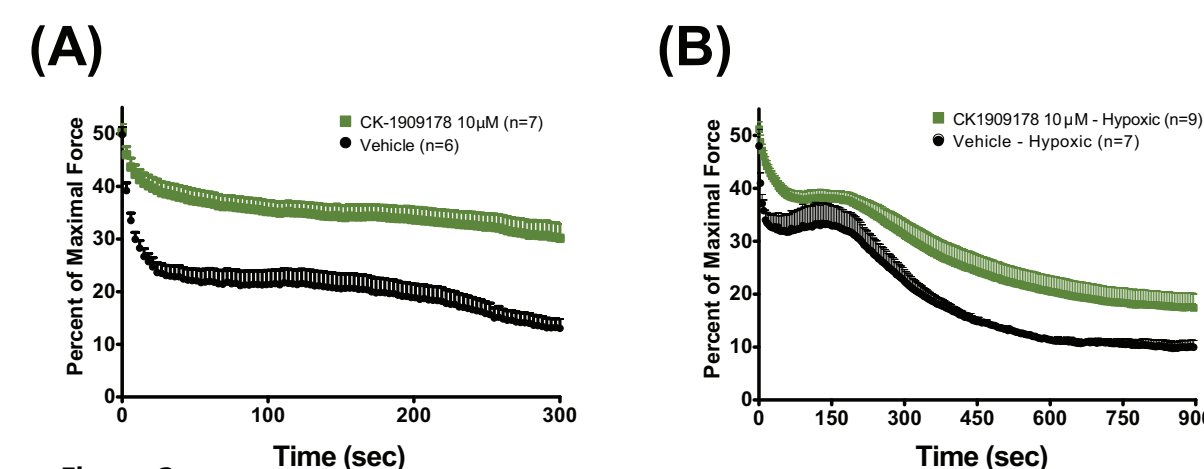


Figure 2:

CK-1909178 decreases fatigue in skeletal muscle preparations *in vitro*, during normal and hypoxic conditions. FDB muscles were pre-incubated for 30 min at 4°C in Krebs buffer at resting tension with DMSO (0.1%) or CK-1909178 (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 3 seconds for 5 minutes or 15 minutes, normoxia and hypoxia respectively.

(A) Graph showing the percent of maximal force over time (+/- S.E.M.). CK-1909178 attenuates low frequency fatigue in fast twitch muscle under normal conditions (oxygen content 58.2% +/- 10.6).

(B) CK-1909178 at 10 μM protects muscle from low frequency fatigue in a low oxygenated buffer (18.3% +/- 3.7%).

In situ Protocol with Frequency Titration

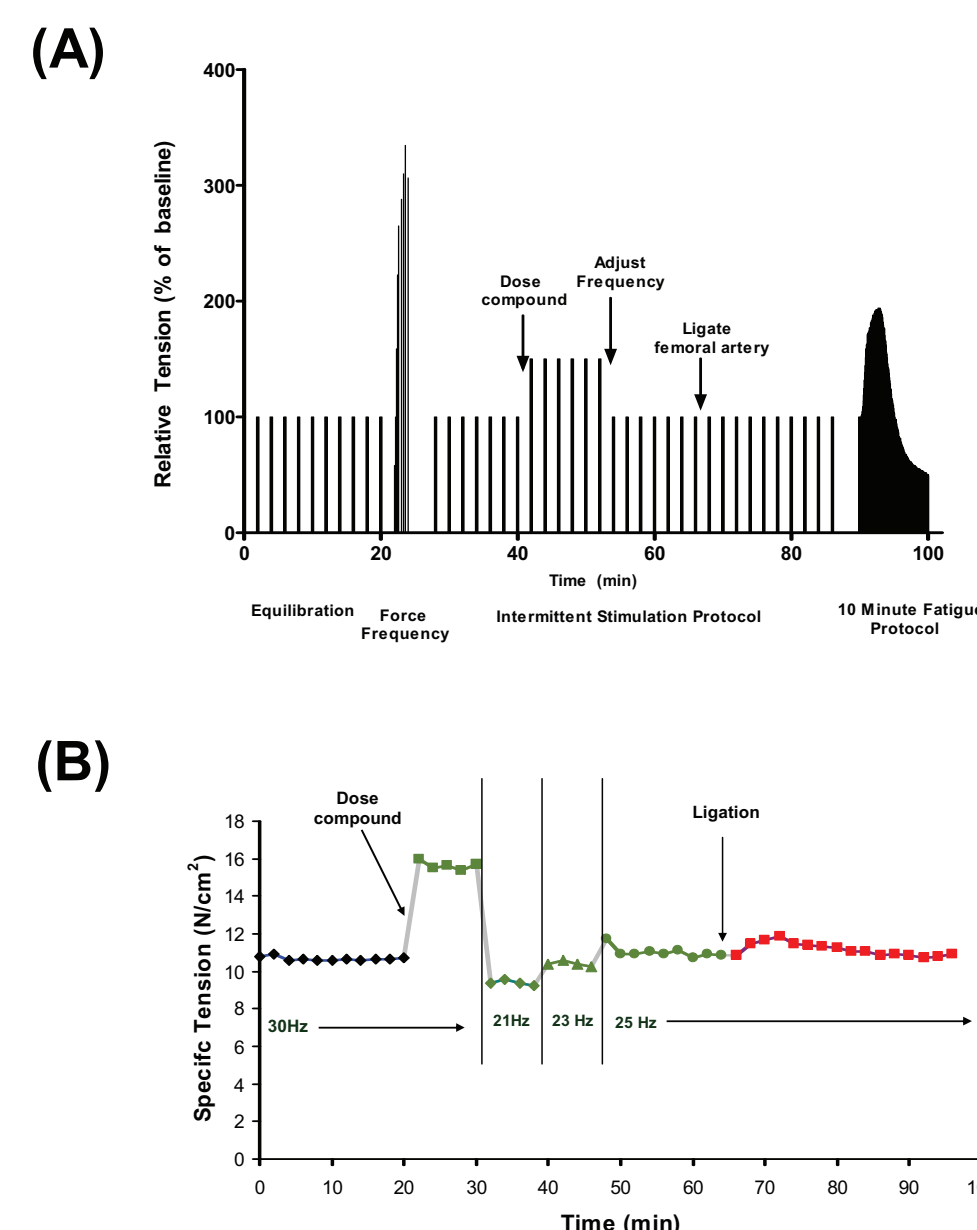


Figure 3:

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 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-compound force level.

CK-1909178 Reduces Fatigue *in situ*

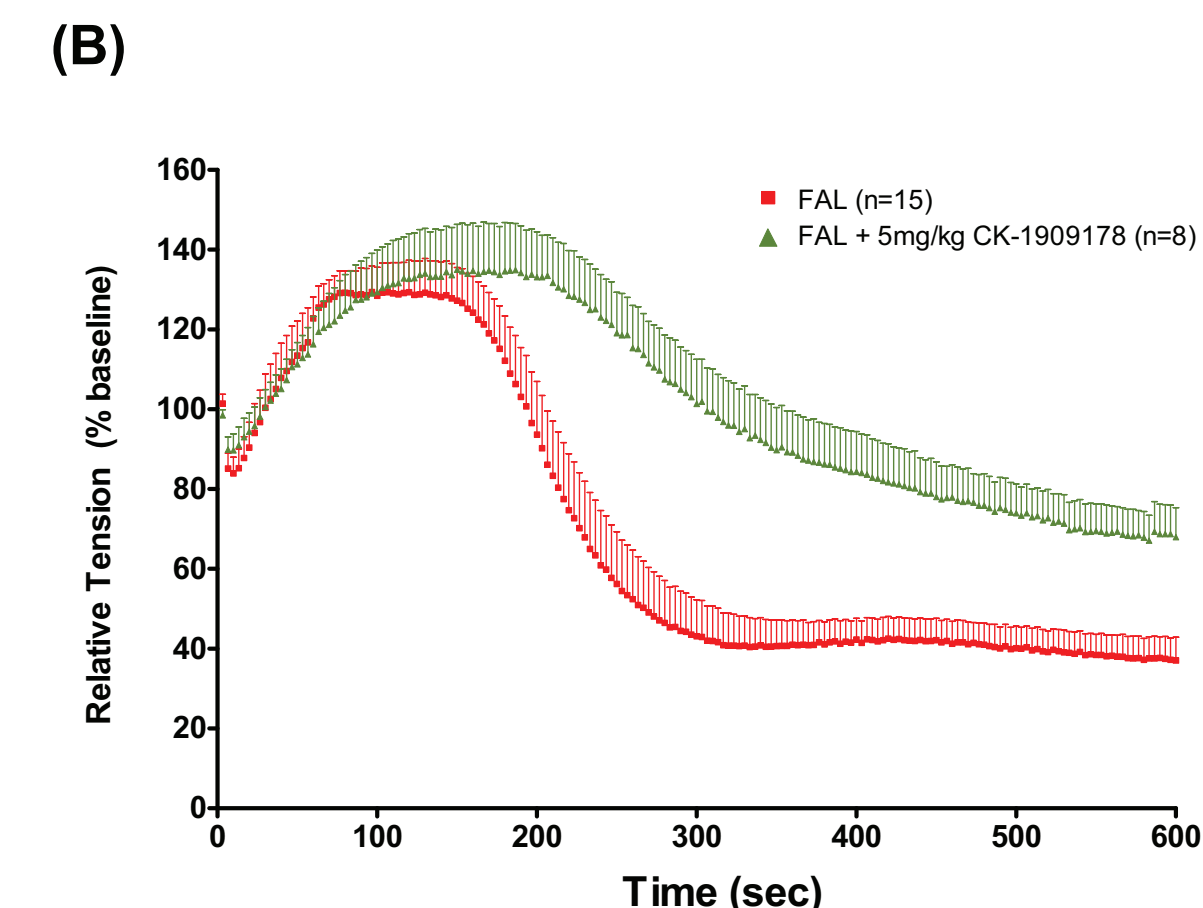
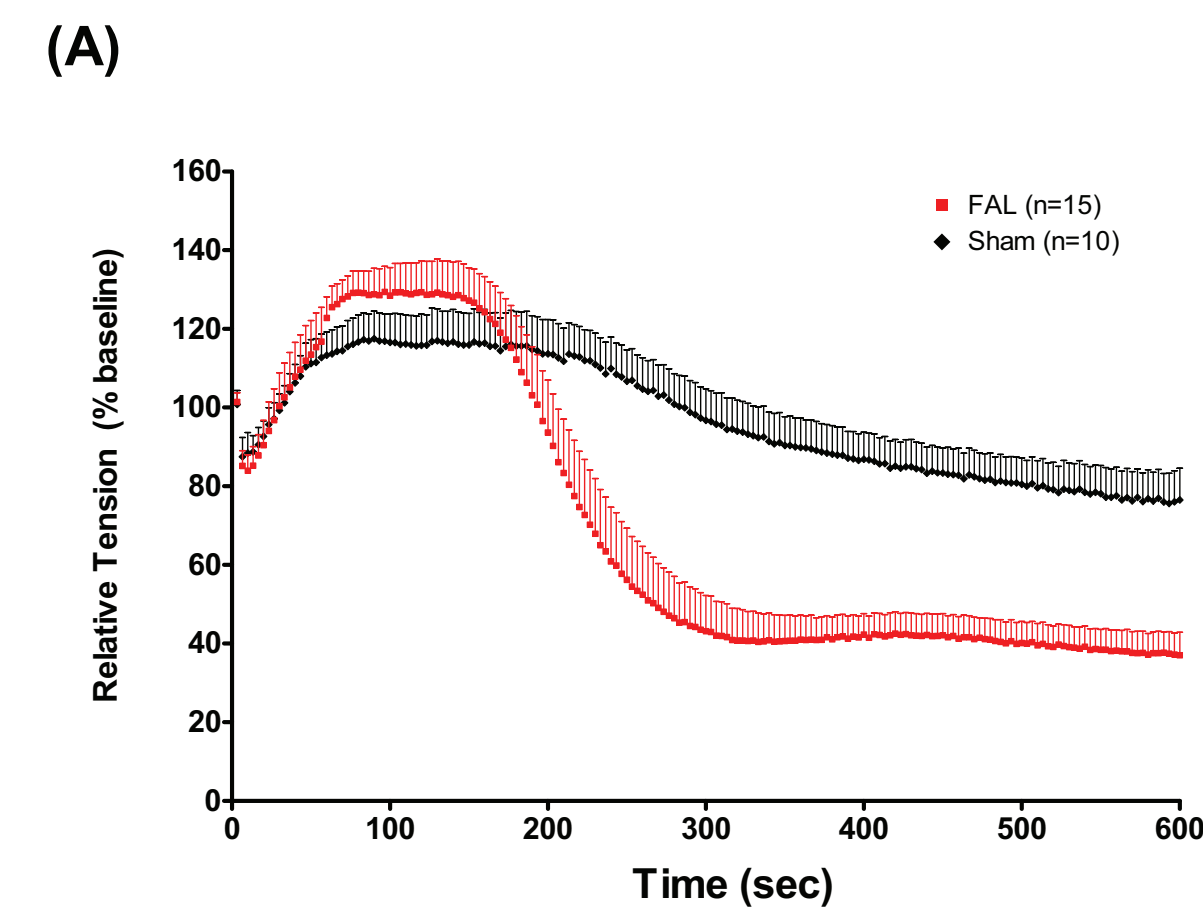


Figure 4:

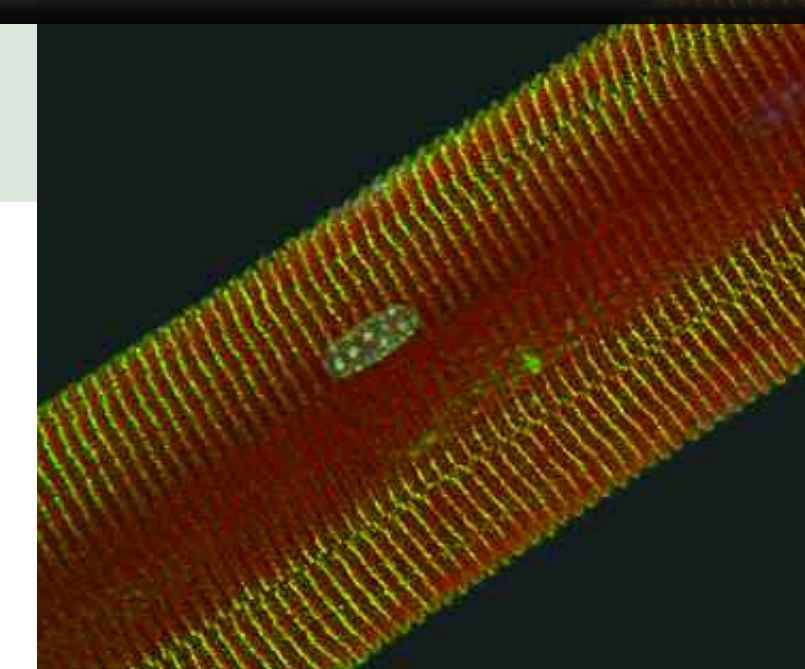
CK-1909178 attenuates fatigue in skeletal muscle with reduced blood flow. Fatigue protocol is intended to be representative of moderate exercise fatigue, not maximal fatigue. EDL is stimulated with 350ms trains every 3 seconds for ten minutes at a frequency that produces the same force as baseline. Vehicle rats are stimulated at 30Hz for entire experiment.

(A) Increased fatigue, as indexed by lower tension generation, occurs in vehicle treated rats with a femoral artery ligation, as compared to sham.

(B) CK-1909178 (average 26 +/- 1.2 Hz) significantly maintains tension in FAL rats as compared to vehicle treated FAL animals.

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CONCLUSIONS

- Skeletal troponin activators increase the calcium-sensitivity of force production in skinned fast skeletal muscles.
- Skeletal troponin activators increase sub-maximal force development in isolated muscle *in vitro*, as well as, reduce overall fatigue in repeatedly stimulated fast skeletal muscle in normal and with hypoxic conditions.
- Skeletal troponin activators increase sub-maximal force development in the EDL muscle of rats after arterial administration of compound. In addition, activators ameliorate fatigue 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-1909178. In skinned muscle fibers, CK-1909178 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, skeletal muscle activators reduces isometric muscle fatigue *in vitro* in normal and low oxygenated conditions. Moreover, CK-1909178 reduces muscle fatigue *in situ* when blood supply is restricted.

These findings suggest functional improvements in skeletal muscle performance and efficiency in conditions marked by muscle weakness by improving the extent of muscle fiber recruitment during physical activity. In addition, findings indicate the potential to ameliorate dysfunction induced by peripheral vascular insufficiency, such as exists in intermittent claudication, when stimulation frequency is adjusted to match tension generation.



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