The Fast Skeletal Troponin Activator, CK-1909178, Increases Skeletal Muscle Force *in vitro* and *in situ*

Ken Lee, James J. Hartman, Richard Hansen, David Clarke*, Aaron C. Hinken, Alex Muci, Bradley Morgan, Vipin Vijayakumar, Zhiheng Jia, Fady Malik, Alan J. Russell

Cytokinetics, Inc., South San Francisco, CA, USA. * Currently at Xoma, Inc., Berkeley, 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 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-1909178 is a fast skeletal troponin activator (See Poster B34) and 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-1909178 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 were also interested in understanding the activity of CK-1909178 in skeletal muscle fibers isolated from fast (type IIa, IIb, IIx/d) vs slow (type I) muscle.

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 $MgCl_2$, 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 relaxing buffer (20 mM MOPS, 5.5 mM $MgCl_2$, 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 $CaCl_2$ 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 NaH_2PO_4, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 137 mM NaCl, 11 mM glucose and 1 mM $NaHCO_3$). 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-1909178 (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-1909178 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 before and after treatment with 10 mg/kg CK-1909178 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).

Results

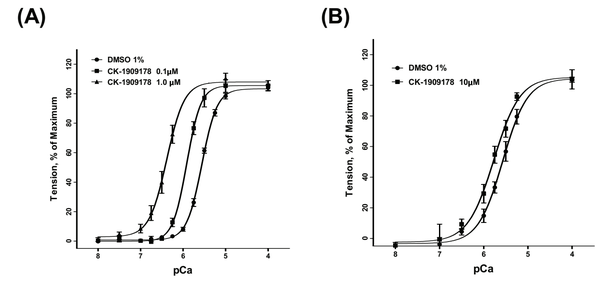


Figure 1. Effect of CK-1909178 on skinned skeletal fibers from **(A)** rabbit psoas muscle (fast skeletal muscle), **(B)** rat soleus muscle (slow skeletal muscle). Single skinned fibers were attached to a model 403A force transducer (Aurora Scientific) at 10°C in a 20 µM MOPS, 132 µM potassium acetate relaxing buffer and force measured after incubation with increasing concentration of buffered calcium and the indicated concentration of CK-1909178 (added as a DMSO stock, final concentration DMSO 1%). Force is plotted against the percent of maximal contraction measured at pCa 4 (n=8, each curve).

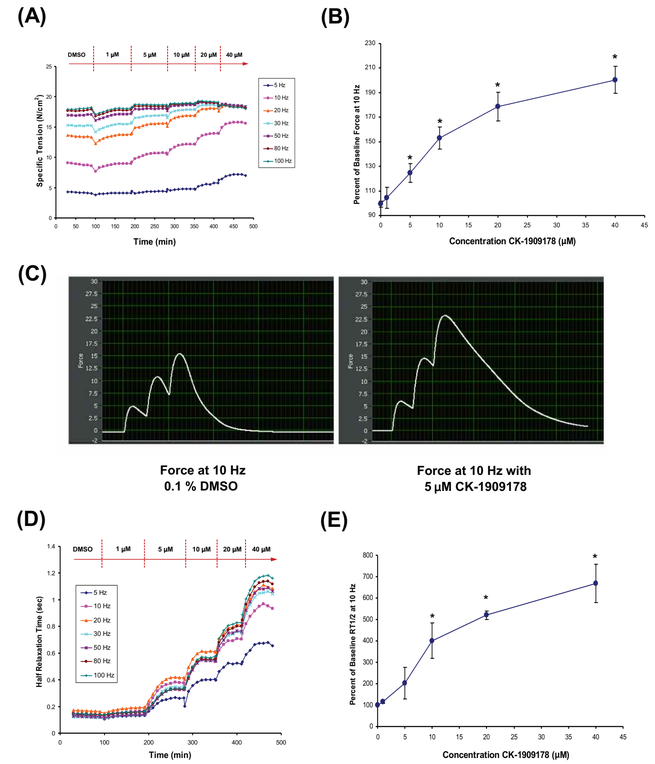
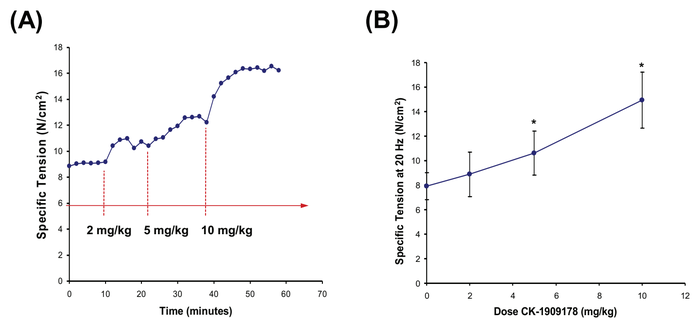
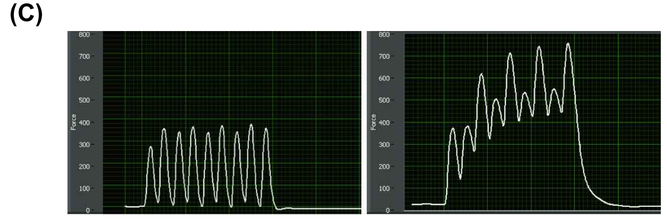


Figure 2. CK-1909178 increases sub-tetanic force and relaxation time in FDB muscle in-vitro. FDB muscles were dissected from the foot of the rat and incubated at 20°C in Krebs buffer. Every 10 minutes, muscles were stimulated with field electrodes at 5-100 Hz and force recorded. **(A)** Result from a single experiment showing the change in specific tension (normalized to the cross-sectional area of the muscle) over time at seven different stimulation frequencies with increasing concentrations of CK-1909178. **(B)** Graph showing the average change in force (mean +/– sd) at 10 Hz vs concentration of CK-1909178 (n=4, * p<0.05 vs DMSO). **(C)** Force trace for a single 10 Hz stimulus at baseline vs treated with 5 µM CK-1909178. **(D)** 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-1909178. **(E)** Graph showing the average change in relaxation time (mean +/– sd) at 10 Hz vs concentration of CK-1909178 (n=4, * p<0.05 vs DMSO).





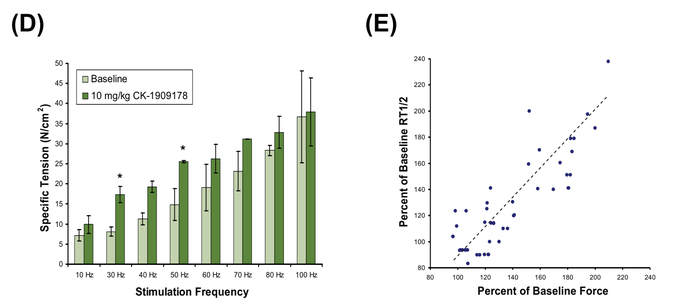


Figure 3. Arterial administration of CK-1909178 increases isometric force in rat EDL muscle *in situ*. The EDL muscle was stimulated every 2 min at 30Hz (1 ms stimuli, 350 ms duration) via the peroneal nerve. CK-1909178 was administered as a 2 min bolus in three cumulative doses up to 10 mg/kg via the femoral artery. **(A)** Real-time results from a single experiment. Force is plotted as specific tension (N/cm²) vs time. **(B)** Average peak specific tension vs cumulative dose of CK-1909178 (* p<0.05 vs vehicle, n=3). **(C)** Force trace for a single 30 Hz stimulus at baseline vs 10 mg/kg CK-1909178. **(D)** Average peak force vs stimulation frequency pre- and post-treatment with 10 mg/kg CK-1909178 (* p<0.05, n=3 vs vehicle). **(E)** Relationship between force and relaxation time after arterial administration of CK-1909178 *in situ*. Elicited force of each 2 minute stimulus throughout the experiment is plotted as a percent of predose baseline force vs half relaxation time (RT1/2), (n=3).

References

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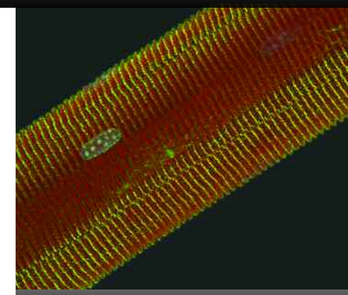

Conclusions

1. The skeletal troponin activator CK-1909178 increases sub-maximal force in fast skeletal rat and rabbit muscle *in vitro*. In living FDB preparations, increases in force are coupled to frequency-independent increases in relaxation time.
2. Skinned slow skeletal muscle fibers are approximately ten fold less responsive to CK-1909178 than skinned fast skeletal muscle fibers, confirming the compound specificity for the fast troponin complex.
3. CK-1909178 increases sub-maximal force 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 troponin activator CK-1909178 in that it increases the sensitivity of skeletal muscle to direct or indirect stimulation which in turn increases muscle force development. We believe that these findings may translate into functional improvements in skeletal muscle performance.

CYTOKINETICS