The Small Molecule Skeletal Sarcomere Activator, CK-1909178, is a Calcium Sensitizer that Binds Selectively to the Fast Skeletal Troponin Complex

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Introduction

Muscle contraction is driven by the cyclical interactions of myosin thick filaments and actin-containing thin filaments. This process is regulated by both nucleotide-dependent conformational changes in the myosin motor and calcium-dependent conformational changes in the thin filament, controlled by the calcium-sensing protein complex troponin. Muscle types differ in their sarcomere components, particularly the isoforms of myosin and troponin, providing specialization to meet their particular physiological requirements.

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.

We have identified a potent troponin activator, CK-1909178, that selectively interacts with the fast skeletal troponin complex, slowing Ca^{2+} dissociation. As a consequence, CK-1909178 sensitizes the sarcomere to calcium, shifting the pCa relationship of both ATPase and force (Poster B33) to the left. We propose a model whereby CK-1909178 sensitizes skeletal muscle by stabilizing the Ca^{2+}-bound conformation of troponin, increasing the amount of time thin filaments spend in the active ("open") state.

Materials and Methods

Reagents: CK-1909178 was synthesized by the Medicinal Chemistry department at Cytokinetics. Triton X-100-skinned myofibrils were prepared using variations on the published methods of Herrmann et al. (rabbit skeletal) or the working protocol from the lab of Dr. John Solaro (bovine cardiac). Proteins were prepared using variations on the methods of Potter (troponin), Smillie (tropomyosin), Pardee and Spudich (actin), Margossian and Lowey (myosin), and Weeds and Taylor (S1 fragment digestion).

Myosin isoform analysis was performed using the glycerol SDS-PAGE method of Talmadge and Roy.

ATPase assays were performed at 22°C using a pyruvate kinase / lactate dehydrogenase-coupled enzyme assay system. Skeletal myofibril reactions were performed in a buffer consisting of 12 mM K-PIPES (pH 6.8), 4 mM $MgCl_2$, 60 mM KCl, 0.5 mM ATP, 0.1 mg/ml bovine serum albumin, 1 mM DTT, 0.5 mM NADH, 1.5 mM phospho-enolpyruvate, 0.6 mM EGTA, and $CaCl_2$ sufficient to give the desired free calcium concentration. Cardiac myofibril and reconstituted sarcomere assays were performed under similar conditions, except $MgCl_2$ was reduced to 2 mM and KCl was omitted. Reconstituted sarcomere assays contained 0.5 µM cardiac S1, 14 µM cardiac actin, 10 µM troponin, and 3 µM tropomyosin.

Isothermal titration calorimetry (ITC) was performed by titrating CK-1909178 (300 µM) into purified rabbit fast skeletal troponin (50 µM) at 10°C using a VP-ITC calorimeter (Microcal). Both reagents were equilibrated in 12 mM K-PIPES, 100 mM KCl, 250 µM $CaCl_2$, 5 mM ß-mercaptoethanol, and 3% DMSO. Binding parameters were evaluated using a single site binding model.

Calcium release from troponin was measured using the method of Rosenfeld and Taylor. Rabbit fast skeletal troponin (10 µM) was preincubated with $CaCl_2$ (20 µM) and either DMSO (0.2%) or CK-1909178 (20 µM) in DMSO (0.2%) in PM12 buffer (12 mM K-PIPES pH 6.8, 2 mM $CaCl_2$, 1 mM DTT) and rapidly mixed with 120 µM Quin-2 in PM12 buffer in a stopped flow apparatus (SF-61DX, TgK Scientific). Quin-2 was excited at 337nm, with emission measured through a 495nm long pass filter.

Results

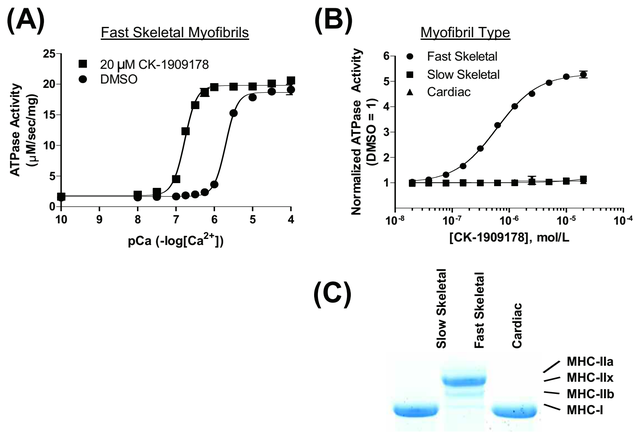


Figure 1. CK-1909178 selectively sensitizes the ATPase activity of skinned fast skeletal myofibrils to activation by calcium. (A) ATPase activity of skinned rabbit fast skeletal myofibrils as a function of free Ca^{2+} concentration (pCa). The calcium sensitivity shifts from a control pCa_{50} of 5.7 (DMSO, circles) to 6.8 (20 µM CK-1909178, squares). Error bars indicate the standard deviation of two reactions in a representative experiment. **(B)** Sensitization is selective for fast skeletal myofibrils. CK-1909178 was titrated into skinned myofibrils from fast skeletal (rabbit), slow skeletal (bovine masseter), and cardiac (bovine heart) muscle at approximately the pCa_{25} for each myofibril type. Error bars indicate the standard deviation of two reactions in a representative experiment. **(C)** Myosin isoform analysis showing the myosin composition of the myofibrils used in (B).

Results (contd.)

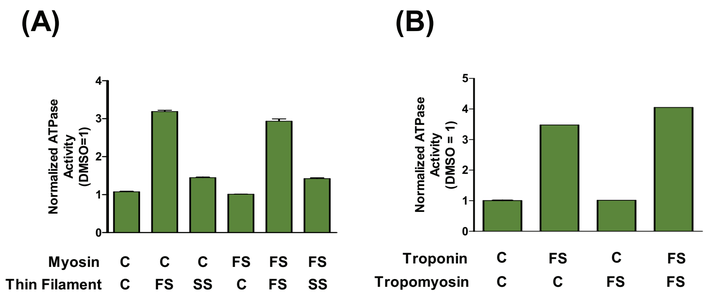
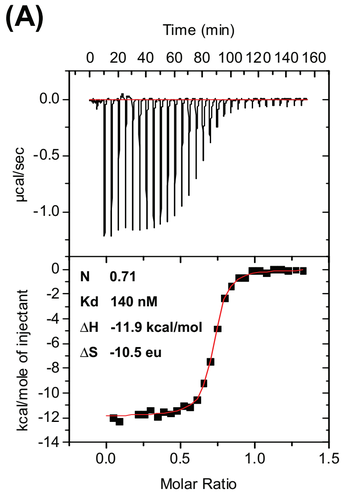


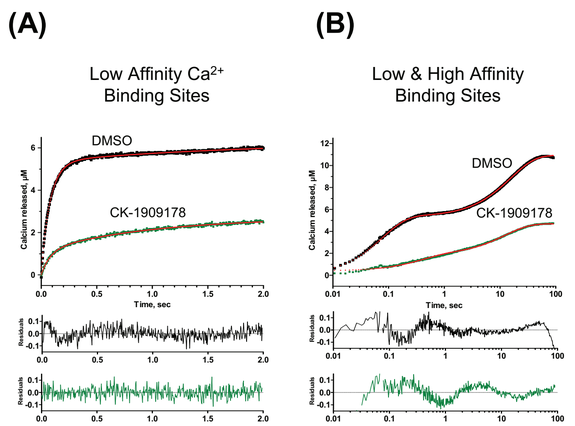
Figure 2. CK-1909178 selectively sensitizes fast skeletal thin filaments to calcium by interacting with fast skeletal troponin. (A) Activation of myosin ATPase is selectively observed with fast skeletal reconstituted thin filaments, regardless of the myosin isoform (fast skeletal or cardiac). The ATPase activity of "reconstituted sarcomere" systems prepared with mixtures of fast skeletal (FS, rabbit), slow skeletal (SS, bovine), and cardiac (C, bovine) components were tested at approximately pCa_{25} for each system. All reactions contained cardiac actin. Error bars indicate the standard deviation of two reactions in a representative experiment. **(B)** Activation requires the presence of fast skeletal troponin. The ATPase activity of reconstituted sarcomere systems prepared from combinations of fast skeletal (FS, rabbit) and cardiac (C, bovine) troponin and tropomyosin were tested at approximately pCa_{25} for each system. All assays contained cardiac actin and myosin (S1). Error bars indicate the standard deviation of two reactions in a representative experiment.



(B)

	Average ± SD
Stoichiometry (N)	0.62 ± 0.13
Kd	120 ± 15 nM
ΔH	-12 ± 1.4 kcal/mol
ΔS	-11 ± 4.6 eu

Figure 3. Isothermal titration calorimetry (ITC) demonstrates direct binding of CK-1909178 to purified fast skeletal troponin. (A) CK-1909178 (300 µM) was titrated into purified rabbit fast skeletal troponin (50 µM) in 3 nmol increments using a VP-ITC calorimeter (Microcal). One representative experiment is shown, with the results of nonlinear fitting to a single binding site model indicated. **(B)** The average data from three separate ITC experiments.



(C)

$$\text{Troponin}(Ca^{2+}) + \text{Quin-2} \rightleftharpoons \text{Troponin} + \text{Quin-2}(Ca2+)$$

Low Fluorescence ⇄ High Fluorescence

$$-A_1 * e^{-k_1 t} + -A_2 * e^{-k_2 t} + C = \text{Dissociation curve}$$

	DMSO	CK-1909178
A_1	5.2 µM	1.1 µM
k_1 (fast)	13 s^{-1}	12 s^{-1}
A_2	1.0 µM	1.6 µM
k_2 (slow)	0.6 s^{-1}	1.0 s^{-1}
C	6.3 µM	2.7 µM

	DMSO	CK-1909178
A_1	5.3 µM	1.4 µM
k_1 (fast)	13 s^{-1}	2.6 s^{-1}
A_2	5.6 µM	2.8 µM
k_2 (slow)	0.06 s^{-1}	0.08 s^{-1}
C	10.9 µM	4.7 µM

Figure 4. CK-1909178 slows release of Ca^{2+} from fast skeletal troponin, in particular the low affinity binding sites. (A) Calcium release from the low affinity (Ca^{2+}-specific) binding sites, monitored using the fluorescence enhancement of Quin-2 upon binding to released calcium. **(B)** Calcium release from both the low (Ca^{2+}-specific) and high affinity (Ca^{2+} and Mg^{2+}) binding sites. Rabbit skeletal troponin (10 µM) was preincubated with 20 µM $CaCl_2$, and 0.2% DMSO (or 20 µM CK-1909178 in 0.2% DMSO) in PM12 buffer followed by rapid mixing with an equal volume of 120 µM Quin-2 in PM12 buffer containing 1.2% DMSO. Released calcium is expressed as the final concentration after mixing (relative to the 5 µM final troponin concentration). Calcium release reactions were analyzed using a double exponential model, and the best fit to the data is shown in table **(C)**.

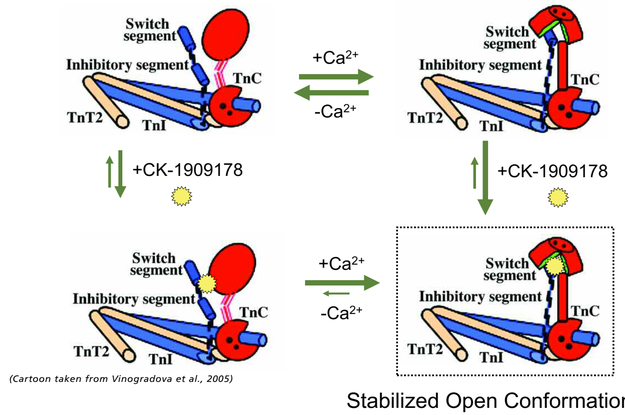
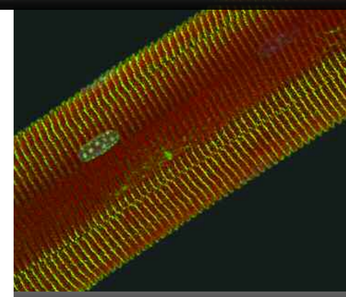


Figure 5. A model for how CK-1909178 could sensitize fast skeletal sarcomeres by stabilizing the open conformation of troponin in the presence of calcium. CK-1909178 is represented by a yellow star; one potential binding site is shown as an illustrative example.



Conclusions

1. The skeletal sarcomere activator CK-1909178 selectively sensitizes the ATPase activity of skinned fast skeletal myofibrils to calcium, without significant activation of myofibrils from slow skeletal or cardiac tissue.
2. Selectivity is due to a requirement for the fast skeletal isoform of troponin; reconstituted sarcomere assays containing slow skeletal or cardiac troponin are not activated by CK-1909178.
3. CK-1909178 binds directly to purified fast skeletal troponin as demonstrated by isothermal titration calorimetry.
4. Calcium dissociation from fast skeletal troponin is slowed by CK-1909178, consistent with its activating effect on myofibrils at intermediate (but not high and low) calcium concentrations.

This compound has also been shown to activate intact fast skeletal muscle sub-maximal force *in vitro* and *in situ* (Poster B33), encouraging further studies of the potential therapeutic uses of troponin activators in diseases of where skeletal muscle weakness plays a role.

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