We have identified a small molecule activator, CK-1213296, of the cardiac myosin ATPase. This agent was characterized in steady state and transient kinetic assays to understand its mechanism of action. CK-1213296 increases the steady-state rate of ATP hydrolysis of purified bovine actin and cardiac myosin subfragment-1, as well as in bovine cardiac myofibrils, where the sarcomere structure is intact. We also find that CK-1213296 activates cardiac myosin selectively as no activation is observed in systems containing rabbit skeletal or chicken gizzard-smooth muscle myosins. Analysis of the individual steps in the enzymatic cycle of cardiac myosin suggests that the actin-stimulated release of phosphate is increased in the presence of CK-1213296. Additionally, we find that no other steps in the enzymatic cycle are affected by CK-1213296. Thus, the enzymatic step governing the weak to strong transition of S1 binding to actin is accelerated without affecting the release from the strongly bound state. This acceleration in the rate of transition from the weak to strong binding state should increase force production and may underlie its ability to improve cardiac contractility in cellular and in vivo models of cardiac function.

**MATERIALS & METHODS**

**Materials**

CK-1213296 was synthesized at Cytokinetics Inc. All proteins used were produced by the protein production group at Cytokinetics Inc.

**Steady state kinetics**

All steady state kinetics measurements were made using an NaDAD coupled assay system by monitoring the absorbance change at 340 nm in 10 mM Pipes, 2 mM MgCl2, 1 mM DTT pH 6.8 (PMSF) buffering system. Assays were run at 25°C unless otherwise specified. All measurements were carried out in a Molecular Devices SpectraMax plate reader. Substrate titration experiments were conducted as described above. Data was analyzed using Grafit to fit the model shown in the text.

**Transient kinetics**

Transient kinetic measurements were carried out on a Hi-Tech SF-61DZ stopped-flow apparatus temperature controlled to 25°C. At least three transient traces were collected and averaged together for each condition. All data analysis was conducted using the KinetQuest software.

ATP binding to bovine cardiac myosin subfragment-1 (S1) was measured by monitoring the intrinsic tryptophan fluorescence of S1. ADP release rates were determined by chasing mannADP from S1 or acto-S1 with a high concentration of ADP. ATP stimulated release of S1 from actin was monitored using a tagging labeled actin and measuring the change in pyrene fluorescence. Phosphate release was monitored using MDMC modified phosphate binding protein (PBP) for S1 in the presence and absence of bovine cardiac actin (1).

**INTRODUCTION**

There is much interest in small molecule agents that can modulate myosin function. Blebbistatin is an example of one such agent, which specifically inhibits the ATPase activity of type II myosins (2). We discovered a new class of agents that activate the ATPase of myosin and improve contractile function in muscle (3). Previously we identified a small molecule agent CK-1213296 (Figure 1), that specifically activates the ATPase activity of cardiac myosin. We have characterized the mechanism of action of this compound using steady state and transient kinetic methods.

**ABSTRACT**

**Figure 1**. CK-1213296 is a member of a substituted tetrazole family of myosin activators (H).

Figure 2. Dose response of CK-1213296 in system (A) composed of purified bovine cardiac S1 (basal system) (B) purified bovine cardiac S1 and bovine cardiac actin. (C) calcium regulated system composed of recombinant CK-1213296. (1213296) suspended in regulated system composed of purified bovine cardiac S1 (crosslinked system). (D) skinned myofibrils that are at a calcium concentration to achieve 70% activation.

Figure 3. Substrate titrations in the presence of CK-1213296 in system (A) purified bovine cardiac S1 and bovine cardiac actin. (B) purified bovine cardiac S1 conversely attached to bovine cardiac actin (C) skinned myofibrils that are at a calcium concentration to achieve 50% activation. (D) purified bovine cardiac S1. Steady state kinetics were analyzed globally using the model shown in the middle of the figure.

Figure 4. Calcium response in the presence of CK-1213296 in system (A) composed of a crosslinked bovine cardiac regulated this filament. (B) skinned bovine cardiac myofibrils (C) F-actin free myosin in the presence of CK-1213296 activates the actin-associated state (D) the actin associated state.

Figure 5. (A) Effect of a single dose of CK-1213296 on actin-activated ATPase of different myosin-IgG. (B) effect of CK-1213296 on (i) skinned muscle. (C) Correspondence actin-myosin kinetic of a 50% of maximal calcium activation. The first two letters denote the myosin source and the number represents the dose of CK-1213296 used. The other letters indicate the presence of CK-1213296 and the activation by CK-1213296 only occurs in cardiac myosin myosin activated by the P1-Beutin used.

Figure 6. Transient state kinetics of (A) ATP binding to (C5) (B) estimation of the ATP hydrolysis rate at the maximal rate of ATP binding. (C) Transient kinetic parameters for ATP binding under single-turnover conditions. (D) Dependence of pyrene acto-S1 dissociation rate on the concentration of ATP. (E) Phosphate release from CK-1213296 single-turnover conditions. (F) Acto- dependent phosphate release for bovine cardiac S1. Concentration of CK-1213296 was kept constant at 50 μM except in phosphate release experiments where the concentration was varied.

**ACCELERATES PRODUCTIVE PHOSPHATE RELEASE AND INHIBITS NON-PHOSPHATE RELEASE**

**ABSOLUTE**

**CONCLUSIONS**

We have demonstrated that the small molecule compound CK-1213296 inhibits the ATPase of myosin by accelerating the rate of ATP hydrolysis of CK-1213296 and does not affect the Ca2+ mobilization and is not the result of interaction with theregulatory maintenance of the actomyosin.

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