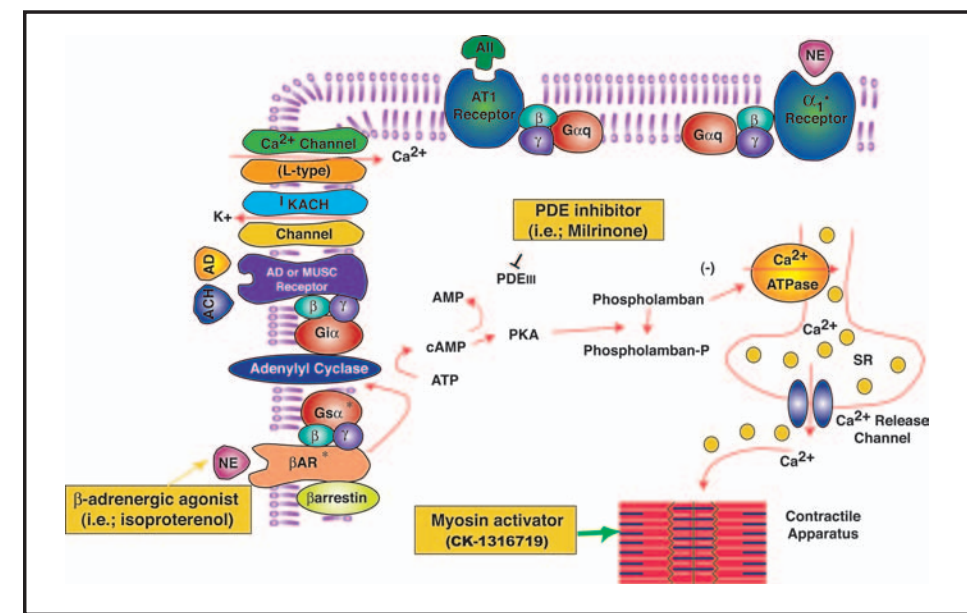


CARDIAC MYOSIN ACTIVATOR CK-1316719 INCREASES MYOFIBRIL ATPASE ACTIVITY AND MYOCYTE CONTRACTILITY IN A RAT MODEL OF HEART FAILURE

Robert L Anderson, Raja F Kawas, Maria V Pokrovskii, Guillermo Godinez, Kenneth H Lee, John Mak, James J Hartman, Bradley P Morgan, David J Morgans Jr., Fady Malik, Roman Sakowicz, Kathleen A Elias
Cytokinetics, Inc. South San Francisco, CA

INTRODUCTION



We have previously reported on a class of small molecule agents, termed myosin activators, that directly stimulate the activity of the myosin ATPase in the cardiac sarcomere. In contrast to current inotropic agents for heart failure (above), myosin activators increase contractility without altering the calcium transient (ref 1-4).

As we have demonstrated that myosin activators increase contractility without increasing the calcium transient in non-failing cells, we now wanted to determine these parameters in cells from animals with heart failure. In heart failure, compensatory mechanisms result in over-stimulation of the β -adrenergic system and induce desensitization to β -adrenergic agents such as isoproterenol or dobutamine. As CK-1316719 activates only cardiac myosin, we hypothesized that myosin activators, unlike calcium altering inotropic agents, would not be desensitized in myocytes and should function equivalently well in cells from heart failure animals or sham controls. Additionally, we predicted that the myosin activator CK-1316719 would increase Ca^{2+} regulated myosin ATPase activity similarly in cardiac myofibrils prepared from heart failure and sham animals.

Heart failure identified *in vivo* and *in vitro*

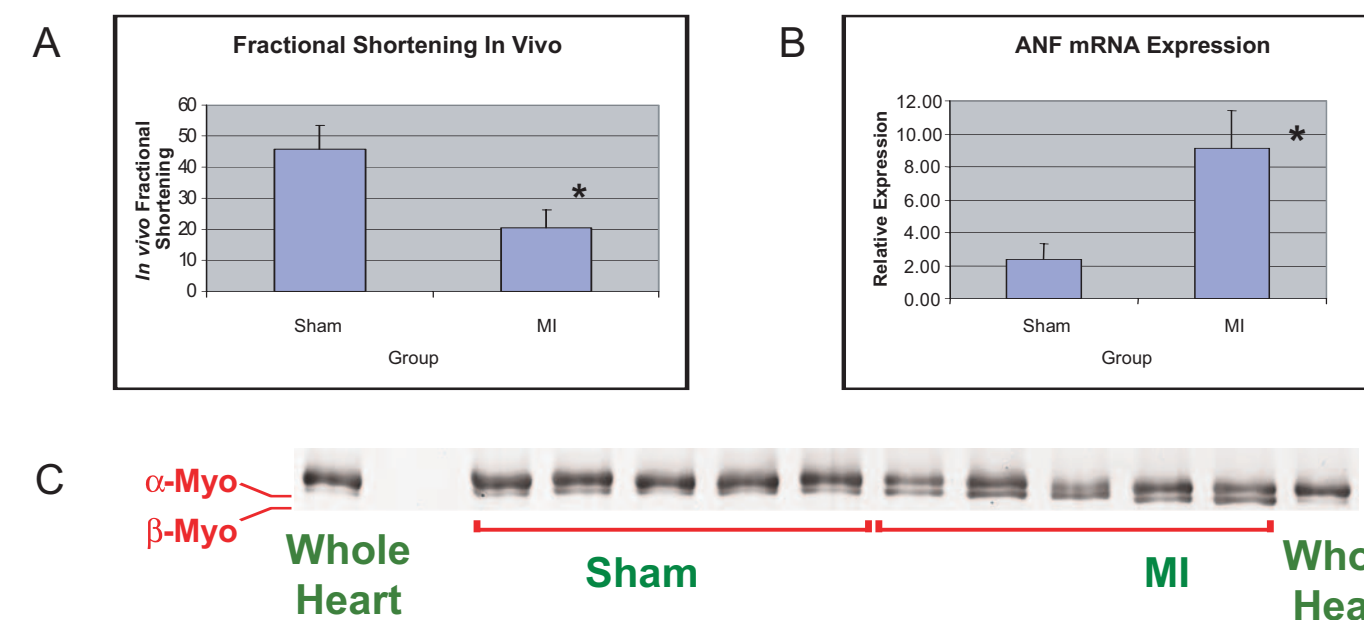


Figure 1. Heart failure was characterized *in vivo* by significant reductions in fractional shortening (A) 12 weeks after myocardial infarction (MI) by echocardiography. *In vitro*, increased left ventricular atrial natriuretic factor (ANF) mRNA expression in MI myocytes (B) and re-expression of the β -myosin isoform in myofibrils (C) demonstrates activation of the fetal gene profile, a characteristic of heart failure.

METHODS

- Myocyte contractility experiments:** Left ventricular myocytes were isolated from adult male SD rats (275-325g) using a collagenase digestion procedure and used within 5 hrs of isolation. Myocytes were warmed in perfusion chambers, perfused with Tyrode buffer and field stimulated at 1 Hz. To determine contractility, myocytes were imaged through a 40x objective and the images were digitized at a sampling speed of 240 Hz. Frame grabber, myopacer, acquisition, and analysis software were obtained from IonOptix (Milton, MA). After an initial 1 min basal contractility period, compounds were perfused for 5 minutes before a 2 min washout period.
- Calcium transient analysis:** Myocytes were loaded with 2 μM fura-2 AM (Molecular Probes) and simultaneous contractility and fura-2 ratios were determined using an IonOptix system modified for fluorescence analysis.
- Cell Analysis:** For each cell, ten or more contractility and calcium ratio transients at basal and after compound addition, were averaged and compared. Contractility average transients were analyzed using the IonWizard analysis program to determine changes in diastolic length, maximum contraction and relaxation velocities, amplitude, fractional shortening (% decrease in the diastolic length; FS) and time to peak. Data were normalized to basal values (basal equals 100%) and expressed as % of basal. The averaged calcium ratio transients were analyzed to determine changes in fura-2 diastolic and systolic ratios and the 75% time to baseline (T_{75}).
- Myofibril assay:** Cardiac myofibrils were prepared using the left ventricle and septum from SD sham or SD rats with heart failure. ATPase assays were performed in a kinetic fashion using NADH coupled enzyme system at $\text{pCa}^{2+} = 6.0$ and 7.0. Rates were normalized to DMSO control.
- Myosin Isoform Gel:** Presence of α - and β -myosin isoforms in the left ventricle and septum from sham and MI rat hearts were determined by loading 10 ng total protein per lane (equating to about 4.5ng of myosin) on an 8% acrylamide SDS gel with 5% glycerol. The gel was run at 80V for 20hrs at 4°C and then silver-stained (Invitrogen). Whole heart from SD rats were used as the standard (ref 5).
- mRNA expression:** Real-time PCR was used to measure expression of ANP and the endogenous reference 18S rRNA. Total RNA was extracted from sham or MI adult rat cardiac myocytes using a Qiagen RNeasy mini kit. The extracted total RNA was treated with DNase and then reverse transcribed using a TaqMan One-Step RT-PCR Master Mix Reagents kit. Real-time PCR reactions were carried out using an ABI PRISM 7900 Sequence Detector (Applied Biosystems). Primers and probes were designed using Primer Express Version 2.0. Relative quantitation of target cDNA sequences was carried out using the comparative CT method. Adult normal rat cardiac myocytes, RNA was used for the 8-point standard curves.
- Heart failure:** Myocardial infarctions (MI) were induced by ligation of the left coronary artery (LCA). Only animals with fractional shortening 3 SD lower than the average ($n=7$) from the sham animals ($n=8$; surgery without tying off LCA) were utilized in experiments. Experiments were performed 12 weeks post-surgery.
- Echocardiography:** The cardiac function (fractional shortening) of sham and MI anesthetized rats (isoflurane) was determined from 2-D M-mode images using an Aplio ultrasound system (Toshiba).
- Statistics:** Data are mean \pm SEM. Statistics were performed using the Students t -test or ANOVA and post-hoc Newman-Keuls as appropriate. $P < 0.05$ was considered significant.
- Reagents:** CK-1316719 provided by the Cytokinetics Chemistry department. All other reagents not detailed above were from Sigma.

Myosin activator CK-1316719 increases fractional shortening equivalently in heart failure or sham myocytes

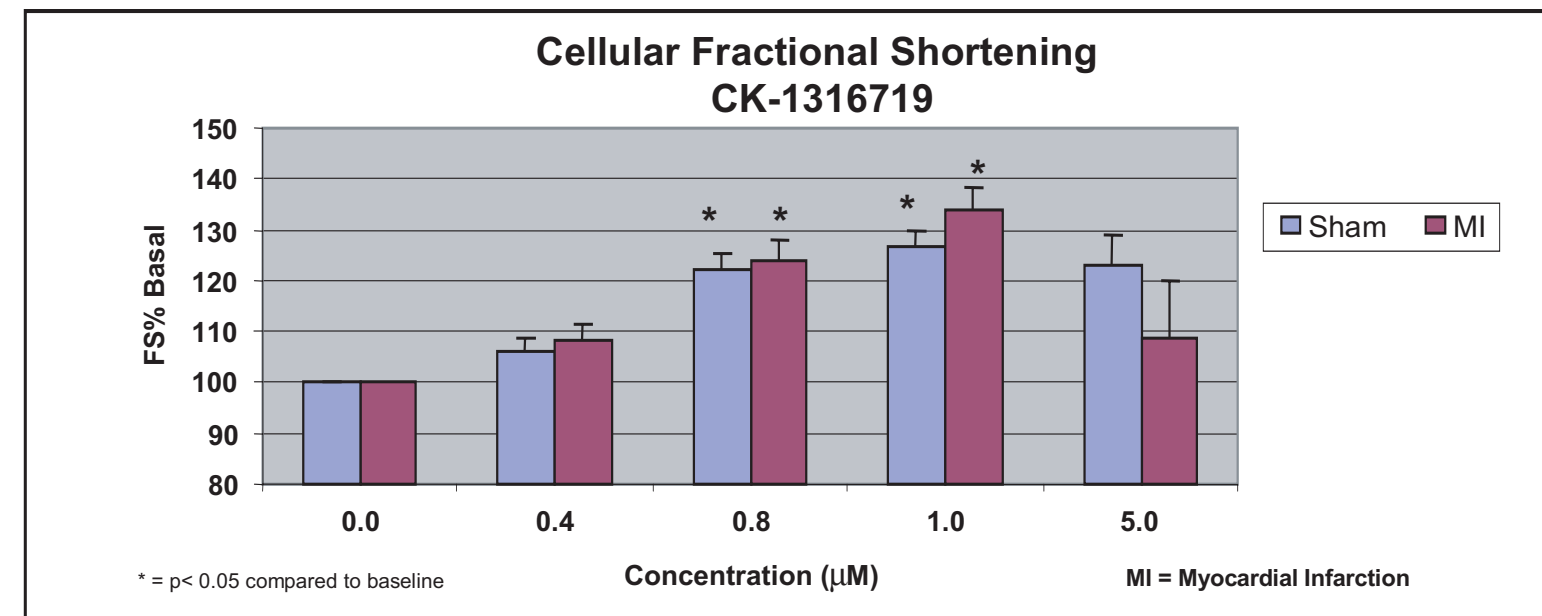


Figure 2. Treatment with CK-1316719 (0.4 – 1 μM) increases fractional shortening in adult cardiac myocytes in a dose responsive manner, equivalently, in both MI and sham cells. At the highest dose fractional shortening is decreased due to changes in diastolic cell length. $N = 10$

Figure 3. In MI cells, response to the β -agonist isoproterenol (20 nM) is significantly truncated compared to sham demonstrating adrenergic desensitization and cellular pathology. Importantly, cells with this limited response to isoproterenol have a full response to the myosin activator CK-1316719 (Figure 2).

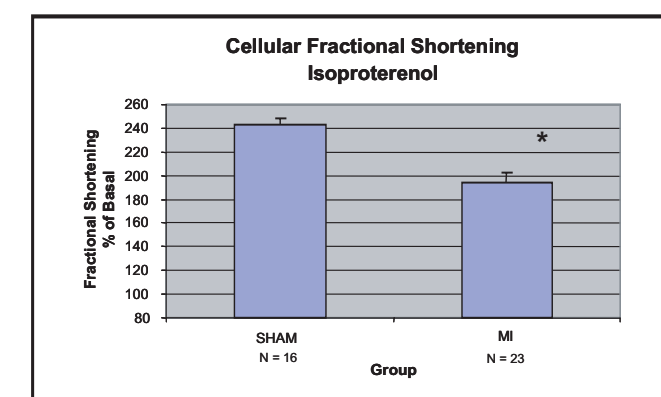


Figure 4. Group data with myocyte parameters from sham and MI cells. Treatment with CK-1316719 increases fractional shortening in a dose responsive manner. In contrast to isoproterenol (ISO) and consistent with a myosin activation MOA, CK-1316719 does not increase contraction or relaxation velocities but does increase the duration of contraction (green box). A decrease in fractional shortening at the highest concentration was observed due to baseline decreases in cell length (red circles).

| Sham μM | Cell Length (% of basal) | Contraction Velocity (% of basal) | Amplitude (% of basal) | Fractional Shortening (% of basal) | Time to Peak (% of basal) | Relaxation Velocity (% of basal) |
|--------------------|--------------------------|-----------------------------------|------------------------|------------------------------------|---------------------------|----------------------------------|
| 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.4 | 99.4 \pm 0.2 | 94.2 \pm 3.0 | 105.2 \pm 2.8 | 105.8 \pm 2.8 | 111.1 \pm 1.1 | 101.5 \pm 4.2 |
| 0.8 | 97.6 \pm 0.3 | 94.1 \pm 3.0 | 119.4 \pm 2.9 | 122.4 \pm 3.0* | 130.4 \pm 2.9* | 113.9 \pm 5.5 |
| 1 | 97.7 \pm 0.3 | 99.1 \pm 2.6 | 123.8 \pm 2.8 | 126.7 \pm 2.9* | 130.5 \pm 2.8* | 114.5 \pm 5.7 |
| 5 | 89.7 \pm 0.7* | 50.0 \pm 3.0* | 110.8 \pm 5.3 | 123.3 \pm 5.5 | 223.2 \pm 5.7* | 68.5 \pm 10.0* |
| 20nM ISO | 98.3 \pm 0.4 | 357.3 \pm 11.2* | 238.5 \pm 5.4* | 242.8 \pm 5.7* | 85.2 \pm 2.2* | 362.4 \pm 22.7* |

| MI μM | Cell Length (% of basal) | Contraction Velocity (% of basal) | Amplitude (% of basal) | Fractional Shortening (% of basal) | Time to Peak (% of basal) | Relaxation Velocity (% of basal) |
|------------------|--------------------------|-----------------------------------|------------------------|------------------------------------|---------------------------|----------------------------------|
| 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.4 | 99.1 \pm 0.2 | 95.6 \pm 3.9 | 107.2 \pm 3.3 | 108.2 \pm 3.3 | 113.4 \pm 2.7 | 101.4 \pm 5.3 |
| 0.8 | 98.1 \pm 0.3 | 99.6 \pm 4.1 | 121.6 \pm 4.1* | 124.0 \pm 4.2* | 126.4 \pm 2.3* | 115.2 \pm 7.0 |
| 1 | 97.3 \pm 0.3 | 95.0 \pm 2.8 | 130.1 \pm 4.6* | 133.7 \pm 4.8* | 135.8 \pm 1.9* | 113.8 \pm 7.0 |
| 5 | 88.0 \pm 1.5* | 46.8 \pm 4.3* | 97.0 \pm 10.9 | 108.9 \pm 11.2 | 212.5 \pm 14.1* | 48.2 \pm 8.7* |
| 20nM ISO | 97.3 \pm 0.5 | 274.2 \pm 19.1* | 189.1 \pm 8.7* | 194.5 \pm 9.0* | 83.6 \pm 11.9* | 243.1 \pm 20.1* |

CK-1316719 increases contractility but not the Ca^{2+} transient in heart failure

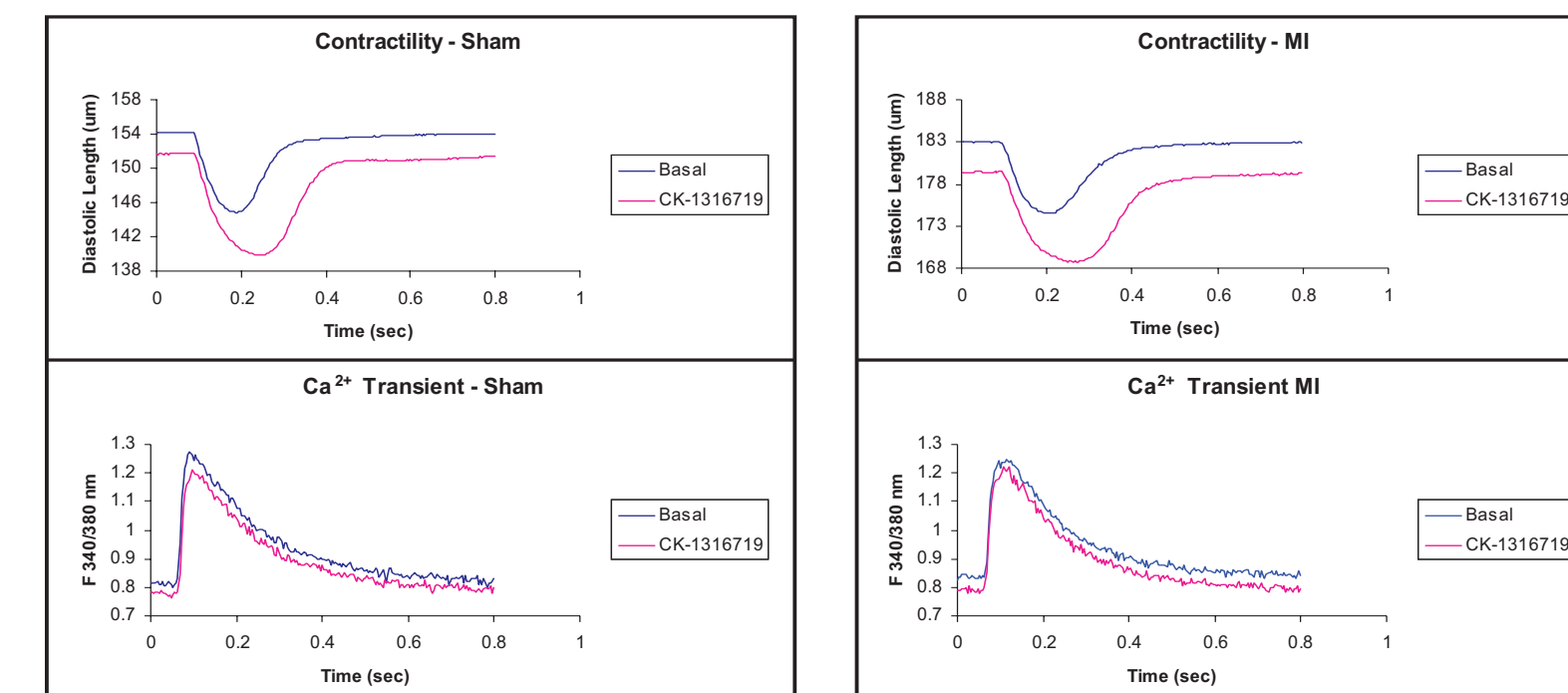
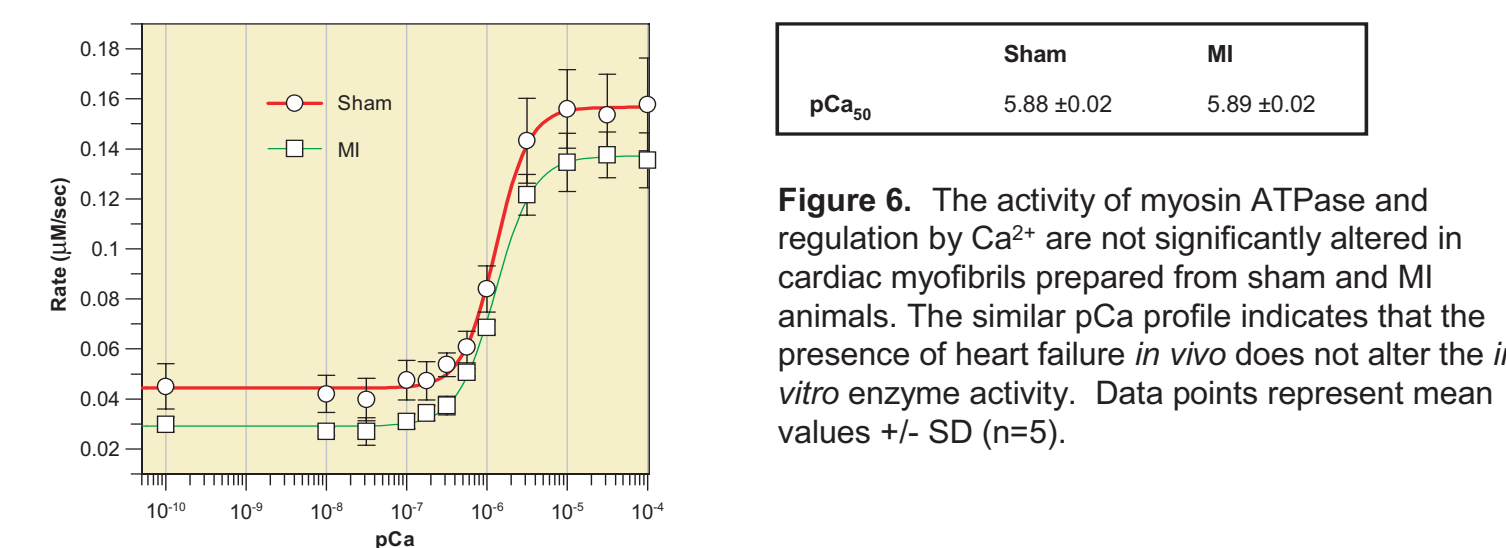


Figure 5. Representative tracings of contractility transient and corresponding calcium transient (fura-2 ratios) before and after treatment with 1 μM CK-1316719 in sham and MI cardiac myocytes (above). In both sham and MI cells, treatment with CK-1316719 increased contractility without increasing the calcium transient. Note the sustained contractility profile characteristic of a myosin activator. Group data are shown below.

| | n | Fractional Shortening (% of basal) | Fura-2 Diastolic Ratio | Fura-2 Systolic Ratio | Time to baseline 75 (% of basal) |
|----------------------------|----|------------------------------------|------------------------|-----------------------|----------------------------------|
| Basal - Sham | 10 | 100% | 0.82 \pm 0.01 | 1.26 \pm 0.01 | 0.27 \pm 0.01 |
| 1 μM CK-1316719 | 10 | 130.1 \pm 4.2 * | 0.79 \pm 0.01 | 1.23 \pm 0.02 | 0.28 \pm 0.01 |
| Basal - MI | 10 | 100 | 0.81 \pm 0.00 | 1.30 \pm 0.02 | 0.29 \pm 0.01 |
| 1 μM CK-1316719 | 10 | 133.5 \pm 5.4 * | 0.79 \pm 0.01* | 1.27 \pm 0.02 | 0.29 \pm 0.01 |

* = $p < 0.05$ compared to baseline

CK-1316719 activates cardiac myosin ATPase in sham and heart failure myofibrils



| | Sham | MI |
|-------------------|-----------------|-----------------|
| pCa_{50} | 5.88 \pm 0.02 | 5.89 \pm 0.02 |

Figure 6. The activity of myosin ATPase and regulation by Ca^{2+} are not significantly altered in cardiac myofibrils prepared from sham and MI animals. The similar pCa profile indicates that the presence of heart failure *in vivo* does not alter the *in vitro* enzyme activity. Data points represent mean values \pm SD ($n=5$).

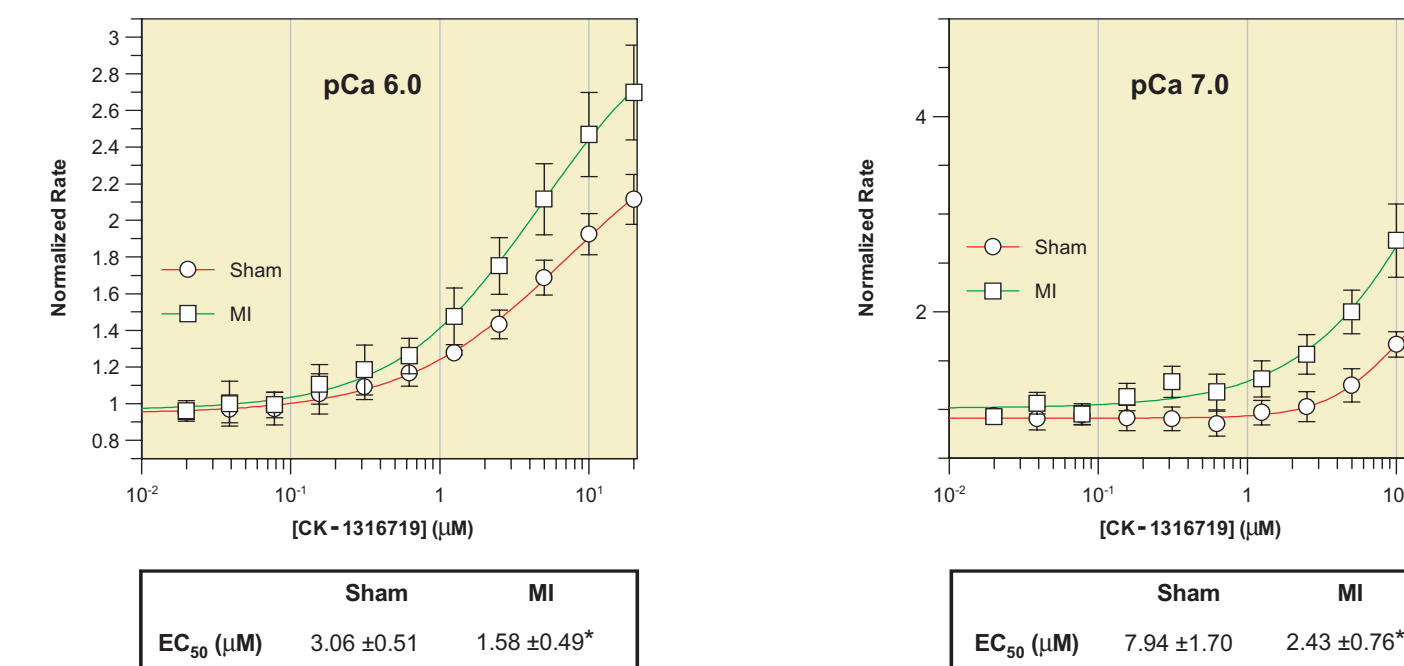
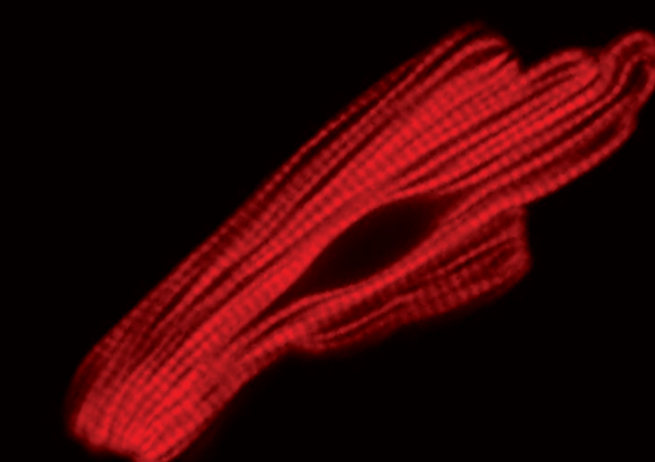


Figure 7. Treatment with CK-1316719 increases myofibril activity from both sham and MI preparations in a dose dependent manner at pCa 6.0 and pCa 7.0. MI preparations are more sensitive to the effect of compound.



CONCLUSIONS

- 1) The cardiac myosin activator CK-1316719 increases contractility in sham and heart failure cardiac myocytes equivalently, without increasing intracellular calcium.
- 2) The cardiac myosin ATPase activity in sham and heart failure myofibrils is similar, supporting myosin ATPase as a therapeutic target in heart failure.
- 3) The cardiac myosin activator CK-1316719 increases cardiac myosin ATPase activity in both sham and MI myofibrils.

These data suggest that myosin activators, such as CK-1316719, may be useful therapeutics in the treatment of human heart failure.

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