A Sensitive Assay for the Basal ATPase Activity of Cardiac Myosin

Ramesh Baliga, Hector M Rodriguez, Donglin Guo, James J Hartman, Daniel W Pierce, Roman Sakowicz, Bradley P Morgan, Fady Malik, Jeffrey T Finer

Cytokinetics, Inc. South San Francisco, CA

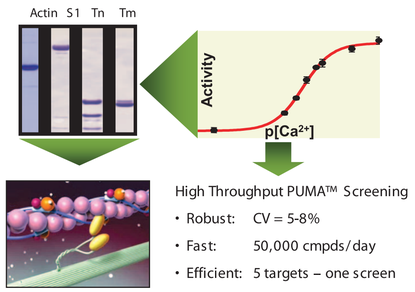
Abstract

We have previously described the use of an absorbance-based kinetic assay for ATPase activity to identify activators of cardiac myosin[1]. One class of these activators has been shown to inhibit the release of inorganic phosphate[2] during basal ATP hydrolysis by cardiac myosin (i.e. in the absence of actin or regulatory components). We used a highly sensitive fluorescence-based assay to test whether the ability to inhibit basal ATPase was correlated with activation of the actin-stimulated ATPase for molecules within this structural class. We found a tight correlation between the two effects. Next, we conducted a screen of a library of ~90,000 compounds to identify additional inhibitors of cardiac myosin basal ATPase activity. Our results indicate that while most of the screening hits also inhibited the actin-stimulated ATPase, a small proportion of them were able to activate the ATPase in the presence of calcium-regulated actin filaments. Thus, we were able to identify compounds that show differing activities depending on the assay context and the opposite effects observed on basal and actin-stimulated ATPase are not unique to a single structural class.

Introduction

Heart failure affects 5 million people in the United States. The lifetime risk of developing heart failure is 1 in 5. Over 80% of men and 70% of women under 65 years old who develop heart failure, die within eight years of initial diagnosis[4]. At Cytokinetics, we have developed novel agents for improving cardiac contractility by specifically activating cardiac myosin. This approach offers several advantages over current agents such as no activation of second messenger signaling, no increase in cardiac myocyte intracellular calcium or in heart rate and no decrease in blood pressure. In addition, this mechanism of action minimally impacts cardiac oxygen consumption and thus improves myocardial efficiency in a dog model[3].

Using a biochemical assay composed of thin filaments reconstituted from actin, tropomyosin and troponins, and cardiac myosin, we have previously reported the discovery of activators of the cardiac myosin actin-stimulated ATPase[1]. The most active compounds belonged to a structural series of substituted benzamides.

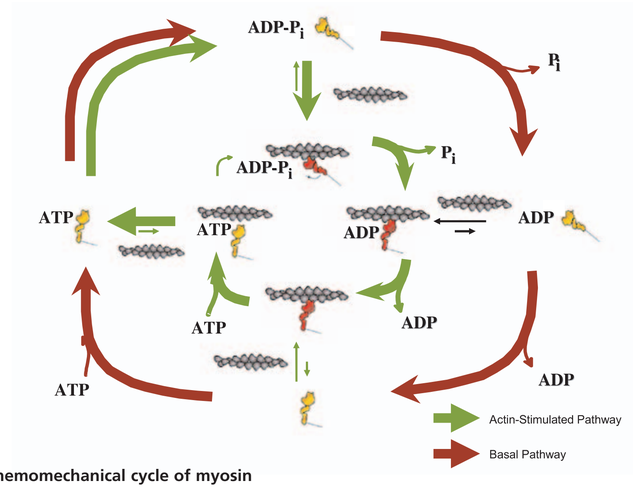


High throughput screen for Cardiac Myosin activators
A reconstituted thin filament assay and an absorbance based kinetic readout of ADP generation was used to identify several activators of the actin-stimulated ATPase activity of cardiac myosin.

Interestingly, a few compounds of this structural series inhibit the release of inorganic phosphate (Pi) in the basal ATP hydrolytic cycle of cardiac myosin[2]. This led us to investigate the following questions:

a) Is inhibition of basal ATPase a general property of this structural series?
b) Are the potencies of basal ATPase inhibition and activation of the actin-stimulated ATPase correlated?
c) With a sufficiently sensitive assay, can we identify other structural families of basal inhibitors that are also capable of activating the actin-stimulated ATPase?

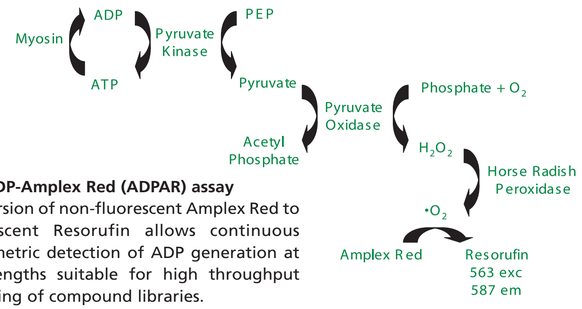
Introduction (continued)



Chemomechanical cycle of myosin
Myosin and actin form various complexes in the presence of ATP resulting in the conversion of chemical energy into force. Myosin can also hydrolyze ATP in the absence of actin. Compounds from the benzamide structural series discovered at Cytokinetics accelerate the rate limiting step of Pi release from actin-bound myosin, but slow down Pi release in the basal ATPase cycle.

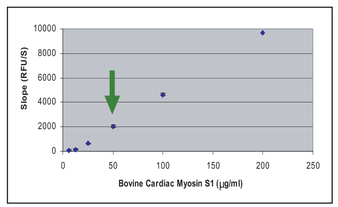
Results

A highly sensitive assay for ADP detection…



The ADP-Amplex Red (ADPAR) assay
Conversion of non-fluorescent Amplex Red to fluorescent Resorufin allows continuous fluorimetric detection of ADP generation at wavelengths suitable for high throughput screening of compound libraries.

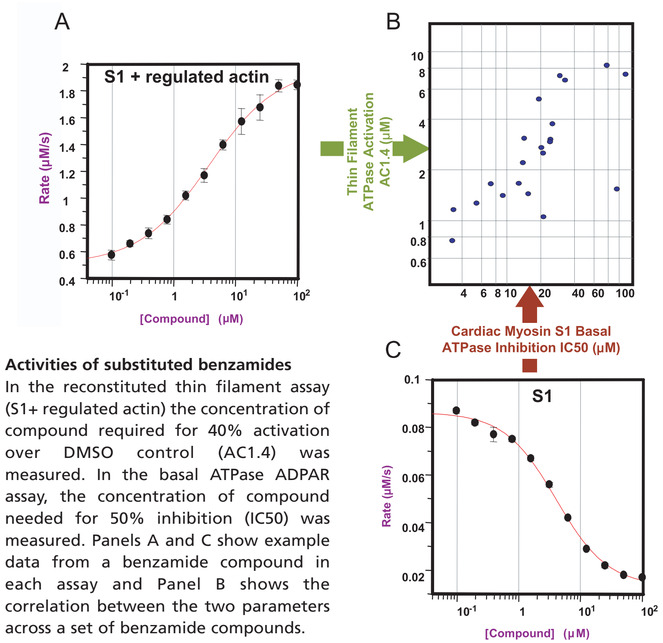
…allows measurement of the low basal ATPase activity of cardiac myosin



ADPAR based myosin basal ATPase assay
The basal ATPase activity of cardiac myosin is very low (Kcat~0.5 s^{-1}), but the continuous fluorimetric assay shown above allows detection of ATPase activity with as little as a few μg/mL of bovine cardiac myosin. 50 μg/mL myosin subfragment-1 (S1) in the presence of 20 μM ATP (~3X Km) was used for the screen (arrow).

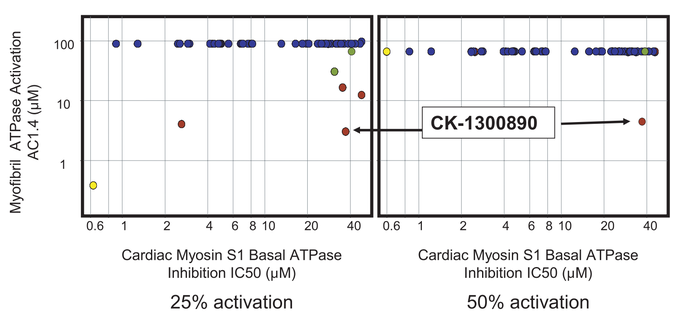
Results (continued)

Basal ATPase Inhibition and Actin-stimulated ATPase are well correlated

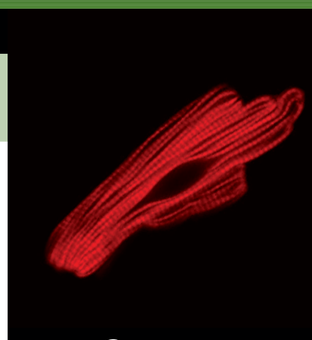


Activities of substituted benzamides
In the reconstituted thin filament assay (S1+ regulated actin) the concentration of compound required for 40% activation over DMSO control (AC1.4) was measured. In the basal ATPase ADPAR assay, the concentration of compound needed for 50% inhibition (IC50) was measured. Panels A and C show example data from a benzamide compound in each assay and Panel B shows the correlation between the two parameters across a set of benzamide compounds.

A screen for basal ATPase inhibitors yields novel activators of myofibrillar ATPase



Potency results from Cardiac Myosin Basal ATPase screen
A majority of the hits from the screen were inhibitors of cardiac myosin ATPase activity measured in a myofibril assay with enough calcium to provide 25% of the maximal calcium activation. Some compounds showed activation of skinned bovine cardiac myofibril ATPase. One of these activators was active at a higher calcium concentration corresponding to 50% activation. Hit series are distinguished by color (blue=inactive).



Conclusions

The basal ATPase activity of cardiac myosin can be measured and utilized for high throughput screening using a highly sensitive fluorescence readout of ADP generation.

There is a strong correlation between activation of the actin-stimulated ATPase and inhibition of basal ATPase for the benzamide family of sarcomere activators.

This correlation allowed us to identify a new series of cardiac myosin activators using a primary screen for myosin basal ATPase inhibitors.

References

1) F. Malik et al, Meeting Abstract, Heart Failure Society of America, 2005.
2) H. Rodriguez et al, Meeting Abstract, Biophysical Society, Annual Meeting, 2006.
3) F. Malik et al, Meeting Abstract, AHA/Keystone 2nd Annual Symposium.
4) Heart Disease and Stroke Statistics, 2005 Update, AHA.

Acknowledgements

We would like to thank the Automation, Corporate Compound Library and Chemistry groups at Cytokinetics for their contributions to this effort.

CYTOKINETICS