Functional analysis of cardiac contractile proteins using in vitro motility assay

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In search of a novel mechanism for improving cardiac contractility, I studied the effect of myosin light chain kinase fragment (MF) consisting of only the light-chain-binding-domain on mechanical properties of actin-myosin interactions using in vitro motility assay techniques. MF increased the sliding velocities of actin filaments on myosin and force generating ability without changing ATPase activity. Kinetics of myosin evaluated by single motor assay (optical trap technique) could account for these functional alterations. To further clarify the molecular mechanism, I propose a conceptual model for the action of MF on actin-myosin interaction. These approaches could contribute to both basic physiology and clinical cardiology.
Key words: Myosin, Myosin Light Chain Kinase (MLCK), Myosin Binding Domain, Laser trap Assay

1. Introduction

Myosin light chain kinase (MLCK) is an enzyme that phosphorylates a serine residue of regulatory myosin light chain (RLC) of myosin and plays an essential role in the control of smooth muscle contraction. In smooth muscle, MLCK was also shown to have a non-kinase stimulatory effect, i.e., MLCK fragments containing the myosin-binding site but lacking the kinase domain increased ATPase activity of unphosphorylated myosin by directly binding to myosin heads without changing the phosphorylation level of RLC[1]. Because MLCK is also expressed in cardiac muscle, we investigated its effect on the motor function of cardiac myosin.

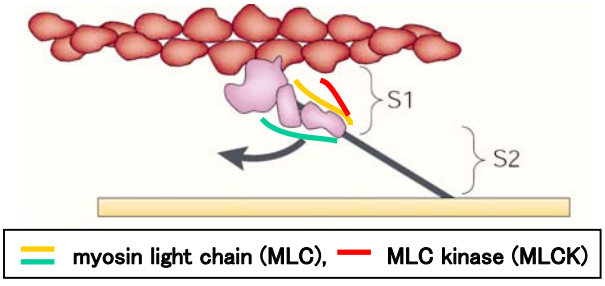

Figure 1. Drawing of myosin

2. Materials and Methods

2.1 Materials

To study the role of myosin-binding domain of MLCK, we expressed a smooth muscle MLCK fragment (MF) containing the myosin biding site but lacking the kinase domain in E. coli (Figure 1). Myosin was extracted from ventricular muscle of Wistar rats. Actin was prepared from rabbit back muscle by the method of Spudich and Watt[6].

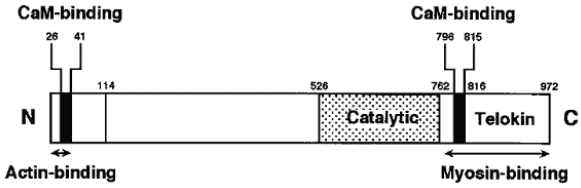

Figure 2. Domain structure of MLCK

2.2 Method

Then we measured phosphorylation level of MLC, actin-activated ATPase activity, actin translocation velocity (VEL), average force generating ability (average force), step size (d), unitary force and ‘on time’ (T_{on}) in the presence (+) and absence (-) of MF. The extent of MLC phosphorylation was confirmed by urea-glycerol polyacrylamide gel electrophoresis. Actin-activated ATPase activity was determined by mixing myosin with various concentrations of actin and measuring released inorganic phosphate. Actin translocation velocity in the in vitro motility assay was measured by the method of Kron and Spudich[2]. The average force measurement was performed as described in the in vitro motility assay system with

some modification. After applying myosin solution, α-actinin solution was infused to the flow cell to create load to the myosin. The following procedure was identical to the in vitro motility assay. The concentration of α-actinin was gradually increased until all the actin filaments were immobilized.

2.2.1 Laser trap system

A single myosin molecule is placed on top of a silica bead and single actin filament with two small beads attached to both ends was lowered by two laser traps, and made to interact with the myosin molecule. When the myosin molecule interacts with the actin filament, the bead which is attached at filament tail is pulled, and the change of bead position is detected by photodiode sensor. The step size, unitary force and 'on time' of a single myosin molecule were measured by laser trap system, the stiffness of the laser trap was compliant (~0.03pN/nm) when measuring the step size and when measuring the unitary force the laser trap was stiffened with feed back (~0.46pN/nm) (Figure 3)[3].

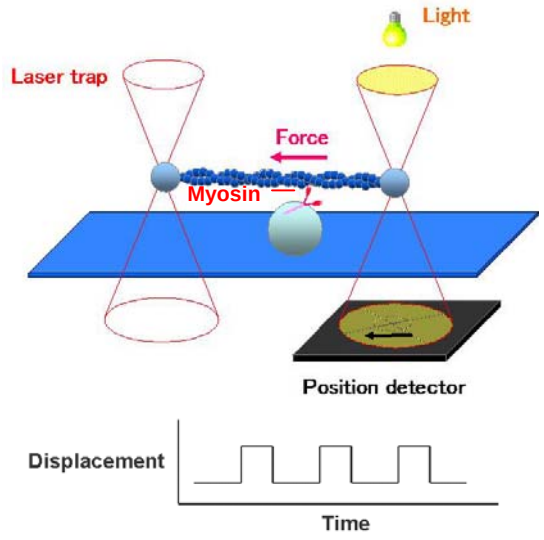

Figure 3. Laser trap system

2.3 Analysis

To derive an unprejudiced estimate of step size and unitary force from our data, we used the technique of "mean-variance" (MV) analysis[4]. In MV analysis a histogram is constructed with each sequential point in the raw data record, the mean, $\langle I \rangle_t$, and sample variance, S_t^2, of that point and the preceeding (W - 1) points are determined, where W is the desired window width in sample points. Mean and variance are defined as follows;

$$\text{Mean ;} \quad \langle I \rangle_t = \frac{\sum_{i=1}^{W} I_{t+i}}{W} \qquad (1)$$

$$\text{Variance ;} \quad S_t^2 = \frac{1}{W-1} \sum_{i=1}^{W} (I_{t+i} - \langle I \rangle_t)^2 \qquad (2)$$

Since variance is reduced when cross-bridge attachment is made, the index of variance can be used to verify Brownian noise from events made by myosin molecule.

3. Result

As shown in the Figure 4. the myosin light chain was not phospholylated by MF while myosin light chain with full-length MLCK was phospholyated.

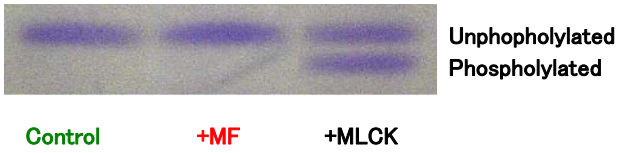

Figure 4. Phospholylation level of MRC

ATPase actibity of cardiac myosin did not show significant difference between control and +MF conditions (Figure 5.).

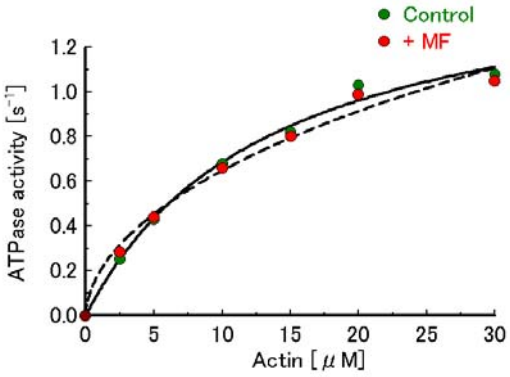

Figure 5. ATPase activity

In +MF condition the actin translocation velocity increased significantly by 22% [5.6 ± 0.5 (+) vs. 4.6 ± 0.4 μm/s (-), $p < 0.01$] (Figure 6.).

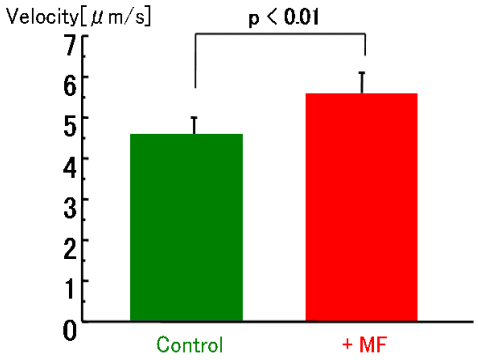

Figure 6. Actin translocation velocity

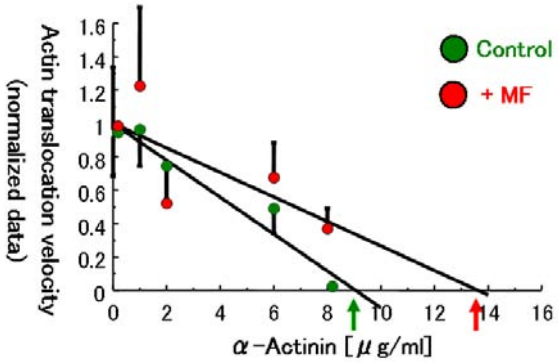

Figure 6. Actin translocation velocity with α -actinin

The results from the assays with kinetic parameters are summarized in the table (Table 1.).

	Control	+MF	Difference
Phospholylation level	Not phospholylated	Not phospholylated	None
ATPase	(Figure 5.)		N/S
Actin velocity (μm/s)	4.6±0.4	5.6 ± 0.5	22%(+)
α -actinin needed to stop actin	9.1 μ g/ml	13.6 μ g/ml	49%(+)
Step size (nm)	14.12±0.71	14.75±0.98	4.5%(+)
Unitary force (pN)	2.02 ± 0.13	2.27 ± 0.26	N/S
T_{on} (msec) Feed back	31.38 ± 2.83	25.67 ± 3.73	5.71msec(+)
T_{on} (msec) No Feed back	27.44 ± 3.73	25.35 ± 2.20	2.09msec(+)

Table 1. Summarized results from the assays

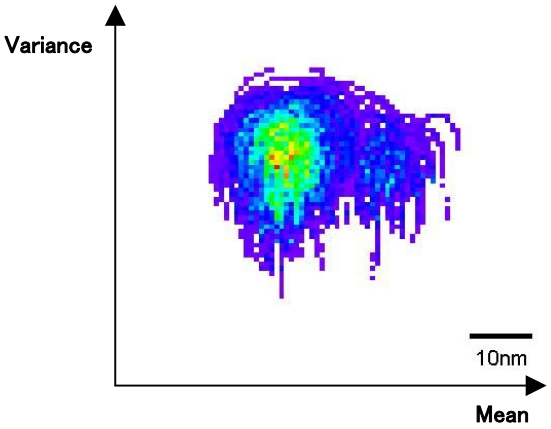

Figure 7. MV-histogram used to measure step size

4. Discussion

The introduction of MF accelerated actin translocation velocity (V) by 22% in the *in vitro* motility assay and we also found that MF causes slight increase in step size (d) and a significant decrease in 'on time' (T_{on}) and also we found an increase in unitary force (although statistically insignificant). In terms of the prevailing framework for the kinetics of actin-myosin interactions, these changes can account for the observed increases in both actin translocation velocity and average force. Relationship between actin translocation velocity (V), step size (d) and 'on time' (T_{on}) are as follows;

$$V = \frac{d}{T_{on}}$$

Relationship between average force, unitary force and 'on time' (T_{on}) are as follows;

Average force=(unitary force)*(T_{on})/(ATPase cycle)

The structure of MF and the experimental confirmation strongly suggests that it binds to myosin regulatory light chain without changing its phosphorylation level. Myosin light chains (MLC) bind to the neck region of myosin subfragment-1 (S1) adjacent to the subfragment-2 (S2). In the lever arm theory, this neck region is considered to transmit and amplify the small conformational change in the catalytic domain accompanying the ATP hydrolysis.

Thus it might well be serving as a putative elastic component of the myosin molecule. We assumed that the binding of MF to the MLC increased the stiffness of this elastic spring. To further explain the underlying mechanisms of this phenomenon, we proposed a model in which i) binding of MF increased the stiffness of the putative elastic component, most likely residing in the lever arm (S1 neck) and ii) myosin undergoes preparatory stroke before attaching actin filament iii) upon binding with actin filament, stored strain energy is released (Fig.3). This model, at least qualitatively, could account for the observed change in the kinetics of actin-myosin interaction in vitro. Finally, the energy saving characteristics of this intervention may be beneficial for the treatment of weakened heart.

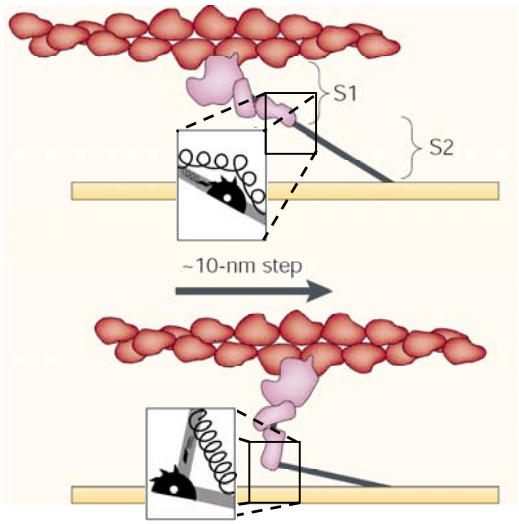

Figure 8. Mechanism of myosin

5. Conclusion

The present results suggest that MF can improve the efficiency of chemo-mechanical energy transduction of cardiac myosin, thus MF has an ideal characteristic required for the agents used for the failing human heart. This unique characteristic of the effect of MF was explained by a novel model of the actin-myosin interaction. I believe this model presents the most feasible mechanism on myosin to date but further study on its validity should be needed.

References

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