Supplemental Data

Smooth muscle myosin phosphorylated at single head shows sustained mechanical activity

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Supplemental Text

ADP release kinetics of the smooth myosin species

An ADP release rate was determined by measuring an apparent dissociation rate of actomyosin induced by a saturating concentration of ATP in the presence of ADP, where the ADP release rate limits the actomyosin dissociation. To determine how much of ADP is required to saturate the active site of the smooth muscle myosin species in the presence of actin, we first determined the affinities of ADP. The HMMs (DHPHMM, SHPHMM, and UPHMM) were premixed with actin and various concentrations of ADP. The mixtures were quickly mixed with 0.2 mM of ATP and the apparent actomyosin dissociation rates were obtained by monitoring the light scattering intensities (Fig. S1, inset). The time courses of the light scattering intensities were well explained by double exponential kinetics and the apparent rate constants were plotted against the ADP concentrations (Fig. S1). The faster rates showed a hyperbolic relationship against ADP and affinities of ADP (K_{ADP}) were determined from the hyperbolic curves. All of the K_{ADP} values were in a range of 4~8 μM (Table S1), which was consistent with the previously reported K_{ADP} value of 5 μM (1). The K_{ADP} values suggest that 0.2 mM is enough to saturate over 95% of the active sites of smooth myosin species with ADP in the presence of actin. The

identities of the slow rates were not clear. Since the slow rates were observed even in the absence of ADP, these rates were probably not relevant to the ADP release event.

In order to determine the ADP release rates, 0.2 mM ADP was premixed with actomyosin and the actomyosin-ADP complex was quickly mixed with various concentrations of ATP. The apparent actomyosin dissociation rates were determined by monitoring the light scattering intensities and these rates were plotted against the ATP concentrations (Fig. S2). Two different rates (fast and slow) were evident in this experiment. The faster rates were fitted with a hyperbolic function and the ADP release rate constants were determined from the V_{max} values (Fig. S2 and Table S2). The ADP release rate constants were not significantly affected by the RLC exchange procedure and the epitope tag of the RLC (Table S2). Similar results were obtained using pyrene-labeled actin (data not shown). Since the slow rates were observed in all of the smooth muscle myosin HMM species, we cannot attribute the slow ADP release rates observed in SHPHMM to the slow actin translocation of SHPMII. Similar slow rates were also reported in a study of myosin IIA, and these slow phases were not completely eliminated by treating actomyosin complex with apyrase (2). The identity of the slow rates was not clear, but it is very likely that these slow rates did not originate from the ADP release event.

Actin binding

An actin binding assay was performed to estimate if the smooth myosin species (DHPHMM, SHPHMM, and UPHMM) bind to actin single-headedly or double-headedly using pyrene-labeled actin (Fig. S3). The fluorescence intensity of pyrene-actin decreases upon strong binding of myosin, and the number of the heads strongly binding to actin was estimated from the fluorescence intensity change by comparing it to that induced by single-headed smooth myosin

fragment. The binding of DHPHMM (pp) to actin decreased the fluorescence intensity of pyrene-actin to a similar extent as S1, suggesting that both heads of DHPHMM bind to actin in the presence of ADP. The decrement of pyrene fluorescence intensity induced by the binding of SHPHMM (pdH and pFdH) was approximately half of that induced by DHPHMM (pp), suggesting that only one of the two heads of SHPHMM preferentially binds to actin in the presence of ADP. The decrement of pyrene fluorescence intensity induced by the binding of UPHMM (dd) was also approximately half of that induced by DHPHMM (pp), suggesting that only one of the two heads of UPHMM preferentially binds to actin in the presence of ADP in our experimental condition. This result can explain the reason why we could not detect the slow ADP release rates of SHPHMM and UPHMM that were expected to be observed dominantly in the light scattering experiment (Fig. S2) if both heads of SHPHMM and UPHMM bind to actin.

Supplemental Materials and Methods

Measurement of ADP release rate

Single mixing experiments were performed in buffer containing 25 mM KCl, 25 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, and 1 mM EGTA at 25^{o}C using a KinTek SF-2001 apparatus with a 75-watt xenon lamp. 0.1 μM HMM was preincubated with 0.3 μM actin and 0.2 mM ADP. The mixture was then rapidly mixed with various concentrations of ATP, and the light scattering intensity at 550 nm was monitored. The ADP release rates were determined from the apparent V_{max} rates of actomyosin dissociation. Similar results were obtained using pyrene actin (not shown).

Actin binding assay

Pyrene-labeled actin was prepared as previously described (3). Single mixing experiments were performed in buffer containing 25 mM KCl, 25 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, and 0.2 mM ADP at 25^{o}C using a KinTek SF-2001 apparatus with a 75-watt xenon lamp. 0.5 μM (myosin head concentration) of smooth muscle myosin S1, DHPHMM (double-head phosphorylated heavy meromyosin)(pp), SHPHMM (single-head phosphorylated HMM) (pdH and pFdH), or UPHMM (unphosphorylated HMM) (dd) was rapidly mixed with 1 μM pyrene-labeled actin, and the decrement in the pyrene fluorescence intensity was monitored using 400 nm long pass filter with the excitation wavelength at 365 nm. The degree of the change in fluorescence intensity was normalized with the fluorescence intensity change induced by DHPHMM.

Supplemental References

1. Cremo, C. R., and Geeves, M. A. (1998) *Biochemistry* **37**(7), 1969-1978

2. Kovacs, M., Toth, J., Nyitray, L., and Sellers, J. R. (2004) *Biochemistry* **43**(14), 4219-4226

3. Kouyama, T., and Mihashi, K. (1981) *European journal of biochemistry / FEBS* **114**(1), 33-38

Table S1. ADP binding affinity

		K_{ADP} [μM]
DHPHMM	pp	8.5 ± 1.4
	pFpF	8.1 ± 1.2
SHPHMM	pdH	5.3 ± 1.3
	pFdH	4.8 ± 0.6
UPHMM	dd	4.9 ± 0.6
	dHdH	3.9 ± 0.7

Table S2. ADP release rate

		ADP off [sec^{-1}]	
		fast	slow (% amplitude)
DHPHMM	pp	28.6 ± 1.6	0.7 ~ 2.6 (4~14%)
	pFpF	30.1 ± 6.1	0.7 ~ 4.6 (8~11%)
SHPHMM	pdH	26.5 ± 2.2	0.8 ~ 2.5 (3~5%)
	pFdH	27.4 ± 1.5	0.6 ~ 1.6 (2~3%)
UPHMM	dd	12.1 ± 0.8	0.7 ~ 1.4 (13~23%)
	dHdH	14.2 ± 1.3	0.7 ~ 1.8 (18~44%)

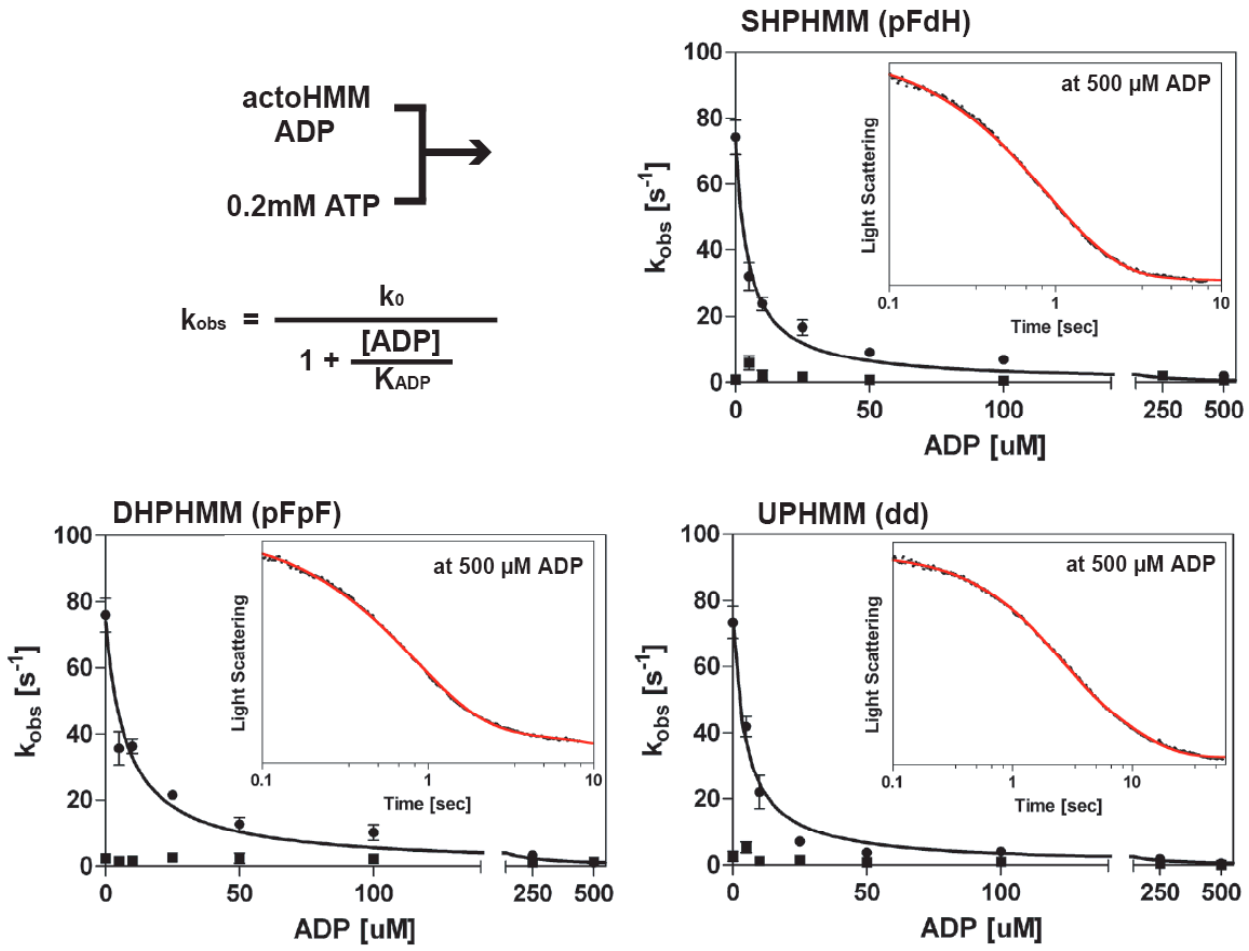

Figure S1. ADP binding affinities. 0.1 μM HMM was preincubated with 0.3 μM actin and various concentrations of ADP, and the mixture was rapidly mixed with 0.2 mM ATP. The ADP concentrations shown in the figures indicate the concentrations after mix. The actomyosin dissociation was followed by monitoring the light scattering intensity at 550 nm. The insets show typical recordings of the light scattering intensity at 500 μM ADP. The time courses of the light scattering signals were best explained by double exponential kinetics (red lines), and both the fast (circle) and the slow (square) apparent rates were plotted against ADP concentration. The ADP binding affinities were determined by fitting the fast rates using the function shown in the figure. The slow rates were observed even in the absence of ADP, and thus may not be relevant to the ADP release reaction. The error bars indicate the standard deviation (n=3~4).

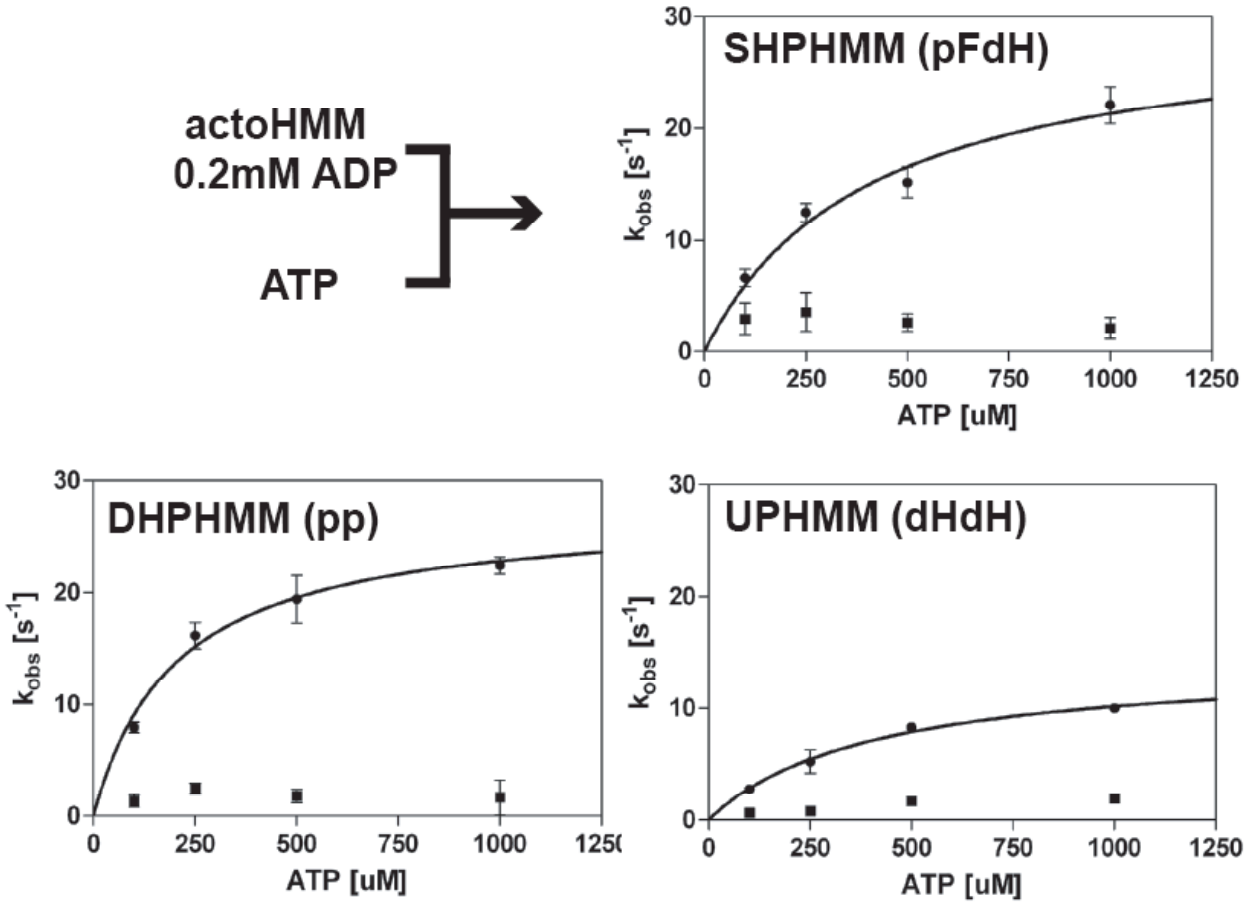

Figure S2. ADP release rates. 0.1 μM HMM was preincubated with 0.3 μM actin and 0.2 mM ADP. The mixture was then rapidly mixed with various concentrations of ATP, and the light scattering intensity was monitored. The time courses of the light scattering signals were best explained by double exponential kinetics, and both the fast (circle) and the slow (square) apparent rates were plotted against ATP concentration. The ADP release rates were determined from the apparent V_{max} values of the fast rates. The error bars indicate the standard deviation (n=3~4).

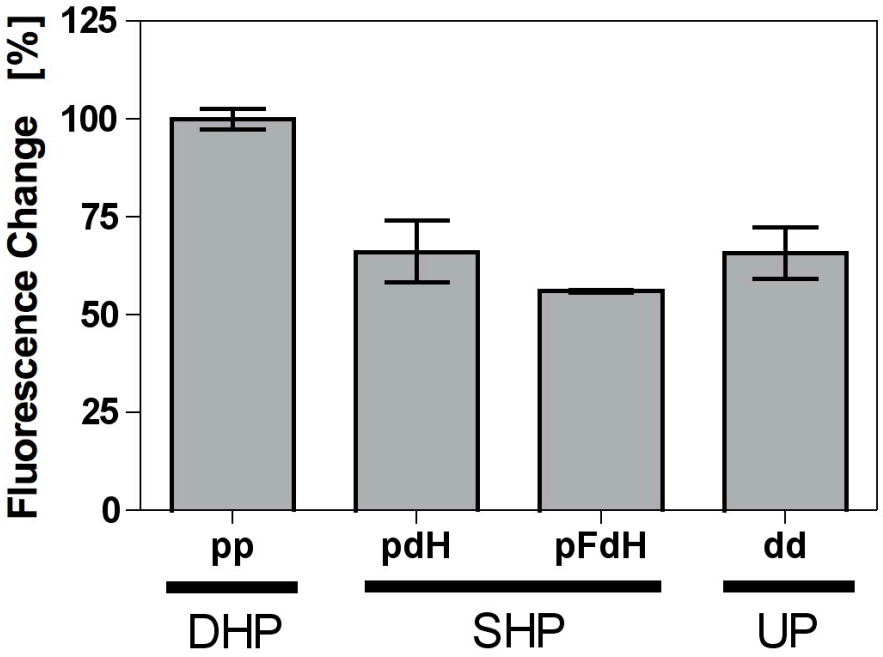

Figure S3. Interaction of DHPHMM, SHPHMM, and UPHMM with actin in the presence of ADP. 0.25 μM DHPHMM (pp), SHPHMM (pdH and pFdH), or UPHMM (dd) was rapidly mixed with 1 μM pyrene-labeled actin, and the decrement in the pyrene fluorescence intensity was monitored. The degree of fluorescence change was normalized with that induced by DHPHMM. The error bars indicate the standard deviation (n=3).