The Small Molecule Smooth Muscle Myosin Inhibitor, CK-2018571, Selectively Inhibits ATP Hydrolysis and Relaxes Smooth Muscle *in vitro*

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Introduction

Smooth muscle myosin is a mechanochemical enzyme that hydrolyzes ATP to generate mechanical force; ultimately all signaling pathways that modulate smooth muscle tone converge on the regulation of this motor protein. We used a high throughput screen to identify compounds that inhibit the ATPase activity of smooth muscle myosin; optimization of the initial hit compounds has resulted in compounds with nanomolar potency. A potent representative of this chemical series, CK-2018571, inhibits the steady-state ATPase activity of human smooth muscle myosin at approximately 10-fold lower concentrations than are required to inhibit non-muscle myosin, the most closely related myosin II and has greater selectivity versus striated muscle myosin IIs. Transient kinetic studies demonstrate that CK-2018571 inhibits the myosin-catalyzed hydrolysis of the γ-phosphate group of ATP, with no effect on nucleotide binding or release from the enzyme. Actin co-sedimentation assays indicate that CK-2018571 stabilizes a weak actin-binding conformation of myosin in the presence of ATP. Consistent with this mechanism, CK-2018571 relaxes skinned rat tail artery muscle tissue. Importantly, this relaxation occurs regardless of whether the skinned muscle has been activated by calcium or by thiophosphorylation of the myosin regulatory light chain, supporting evidence that CK-2018571 relaxes smooth muscle tissue by direct inhibition of activated smooth muscle myosin. The ability of CK-2018571 to relax intact tracheal smooth muscle and aortic ring preparations suggests this mechanism may prove useful in diseases of smooth muscle hypercontractility, such as hypertension and asthma.

Materials and Methods

Biochemical Assays:

Assays were performed in low salt PM12 buffer (12 mM K-Pipes, 2 mM $MgCl_2$, pH 6.8).

Steady-state ATPase activity was measured using a spectrophotometric assay that couples ADP production to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase while regenerating ATP. ATPase rates were measured in the presence of actin and 250 μM ATP (>5-10-fold above the $K_{M,ATP}$). Hydrolysis rates were normalized using reactions containing an equivalent amount of DMSO.

Myosin binding was measured by depletion of soluble myosin from binding reactions using smooth muscle myosin S1 fragment (chicken, recombinant) and 5 μM bovine cardiac actin. ATP and ADP were included at 1 mM where indicated. Reactions were allowed to equilibrate for 15 minutes prior to centrifugation (540k x g, 30 minutes), however ATP was added just prior to centrifugation to minimize hydrolysis. Supernatants were analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue.

Stopped-flow (Hi-Tech SF61DX2) and quench-flow (Biologic SFM/400) experiments were performed at 25°C using smooth muscle myosin S1 fragment (recombinant, chicken). Mant-ATP binding and Mant-ADP release were monitored using fluorescence (λex=360 nm and λem=400 nm). ATP hydrolysis was monitored using malachite green to quantify total acid-labile phosphate.

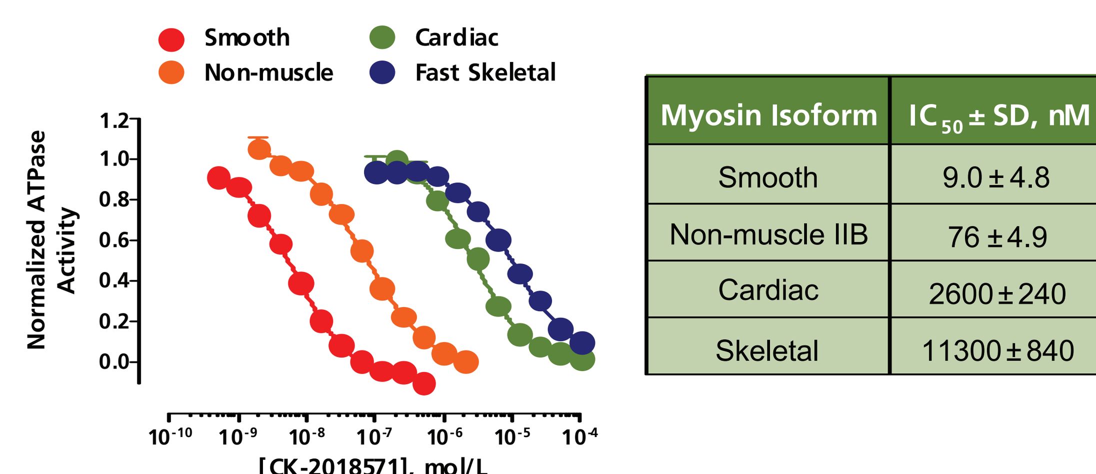
Tissue Assays:

Skinned Ring Assay: Endothelium-denuded rat tail artery segments were cut into 3-mm helical rings, mounted on an isometric force transducer with a resting tension of 0.5 g, and incubated for 30 minutes at room temperature in normal H-T buffer. Tissues were skinned by incubation with skinning solution containing 1% Triton X-100 for 1 hour at room temperature. CK-2018571 was added to the tissue for 15 minutes, followed by addition of solutions with increasing calcium. The force generated at the plateau of each corresponding pCa condition was recorded, and data were presented as a percent change from the baseline values (Wilson *et al.*, 2002).

Thiophosphorylation Assay: Triton-permeabilized preparations were incubated in rigor solution containing ATP-γ-S (1 mM) for 10 minutes CK-2018571 was added 15 minutes before addition of ATP. ATP-induced contraction was measured for 60 minutes and the relaxation was expressed as percentage of the maximum force.

Results

CK-2018571 Selectively Inhibits The ATPase Activity of Smooth Muscle Myosin



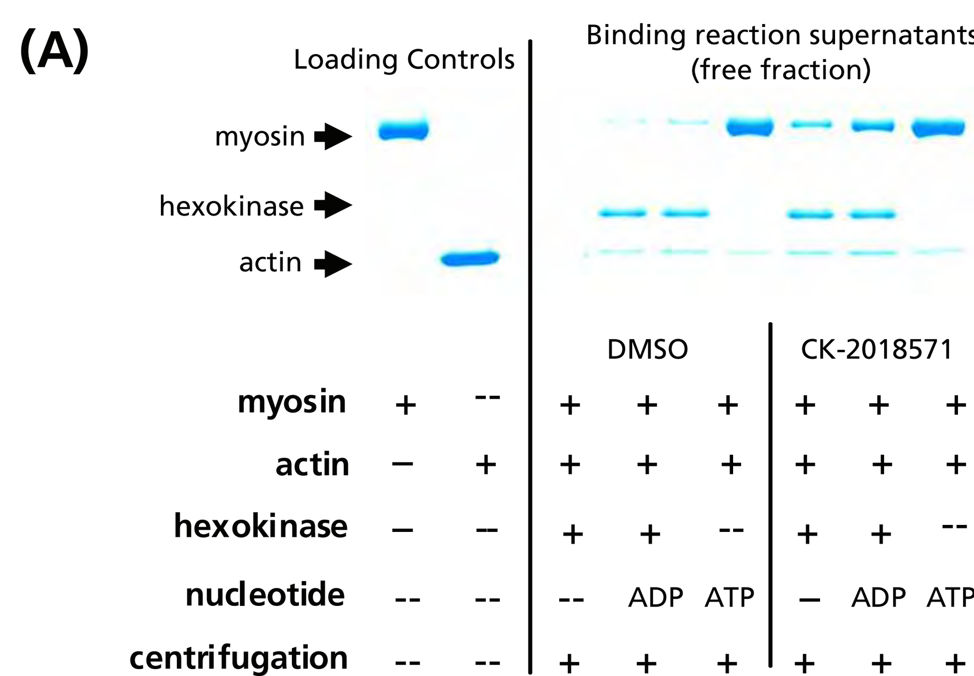
Myosin Isoform	IC_{50} ± SD, nM
Smooth	9.0 ± 4.8
Non-muscle IIB	76 ± 4.9
Cardiac	2600 ± 240
Skeletal	11300 ± 840

Figure 1:

CK-2018571 selectively inhibits the steady-state Mg^{2+}-ATPase activity of smooth muscle myosin. S1 fragments of recombinant human myosin IIs were assayed in low salt buffer in the presence of actin and 250 μM ATP.

CK-2018571 Promotes Weak Actin-Binding

(A)



(B)

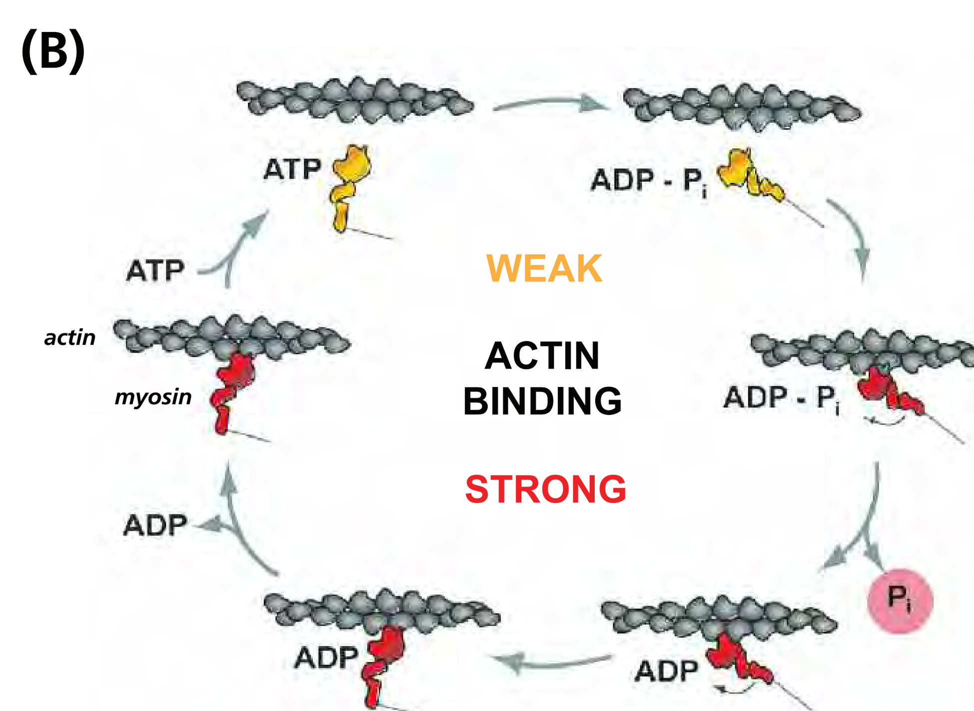


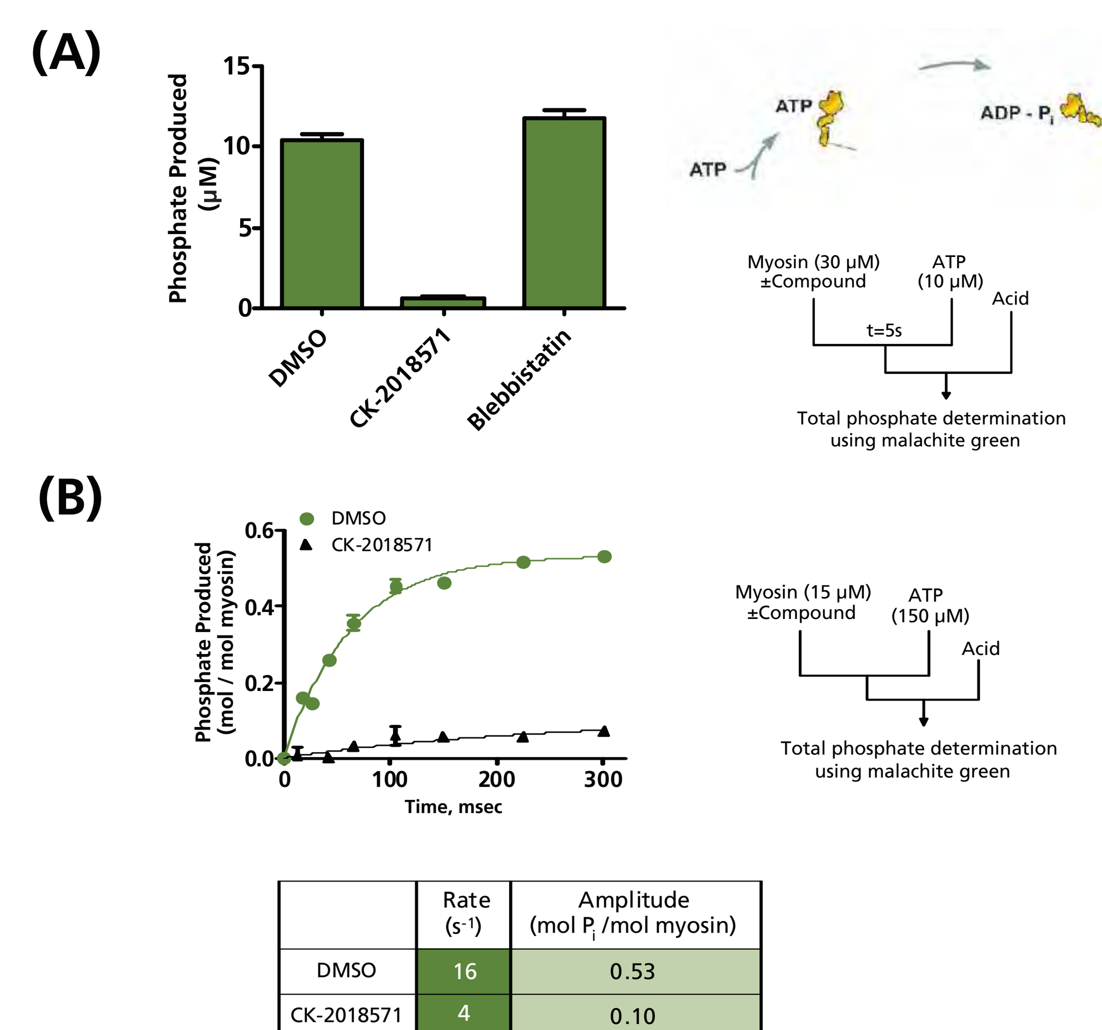
Figure 2:

CK-2018571 inhibits smooth muscle myosin in a weak actin-binding state.

(A) Characterization of myosin affinity by actin cosedimentation. Strong actomyosin binding is revealed by depletion of soluble smooth muscle myosin (recombinant S1 fragment, chicken) upon centrifugation in the presence of actin and various nucleotides. Total amounts of myosin (3 μM) and actin (5 μM) are indicated by the loading controls (left), while unbound myosin is shown in the binding reaction supernatant fractions (right). Hexokinase is included in the no nucleotide and ADP reactions to deplete residual ATP.

(B) The myosin chemomechanical cycle, indicating weak and strong actin binding states.

CK-2018571 Slows ATP Hydrolysis ...



	Rate (s^{-1})	Amplitude (mol P_i /mol myosin)
DMSO	16	0.53
CK-2018571	4	0.10

Figure 3:

CK-2018571 slows the chemical cleavage of ATP by smooth muscle myosin.

(A) Measurement of phosphate burst magnitude under single turnover conditions.

(B) Determination of the ATP hydrolysis rate using rapid mixing quench flow.

Without Significantly Slowing ATP Binding or ADP Release

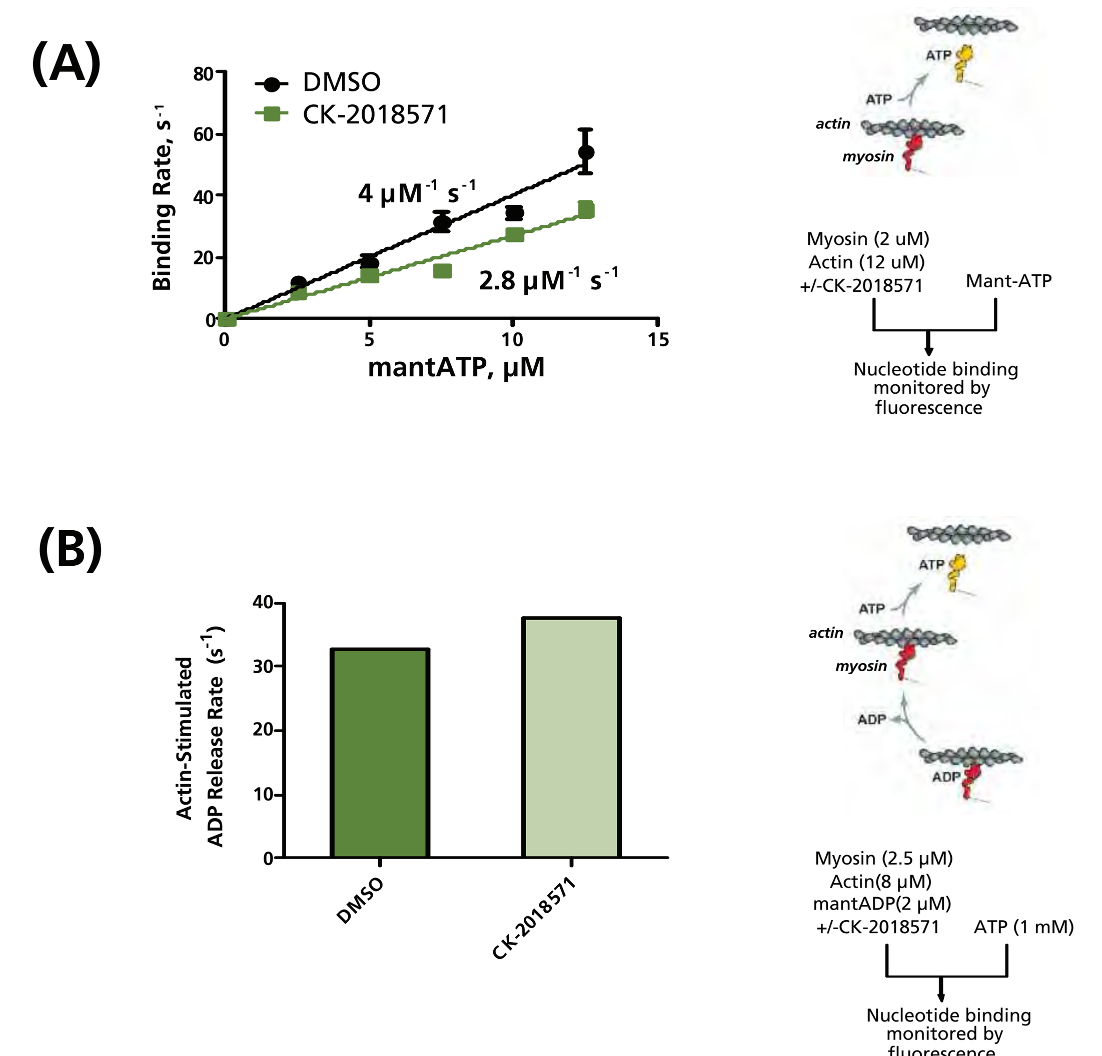


Figure 4:

Effects of CK-2018571 on **(A)** mantATP binding and **(B)** actin-stimulated mantADP release. CK-20187571 was present at saturating (6 μM) concentrations.

CK-2018571 Relaxes Smooth Muscle Tissue *in vitro* ...

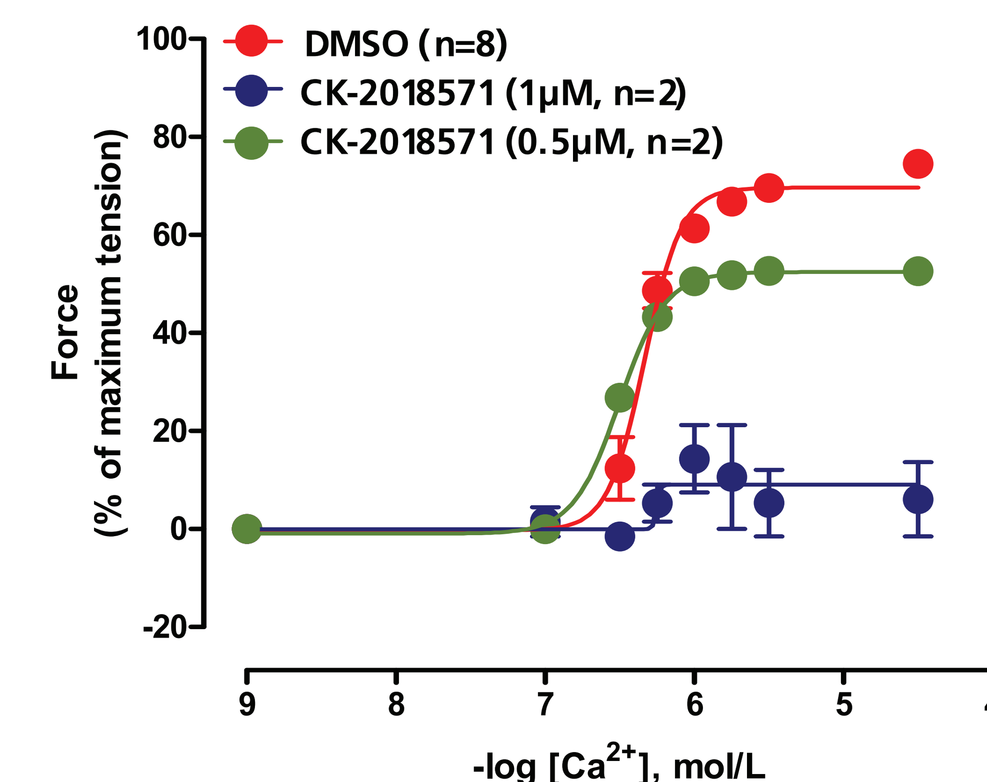


Figure 5:

CK-2018571 inhibits Ca^{2+}-dependent force production in skinned caudal artery rings from Sprague Dawley rats.

Independent of Calcium Activation

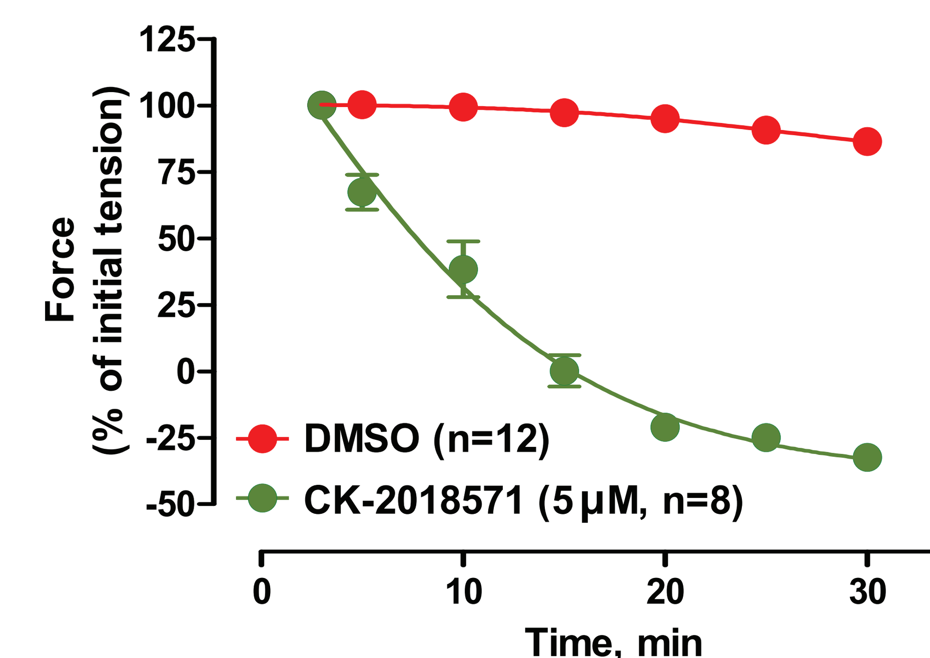
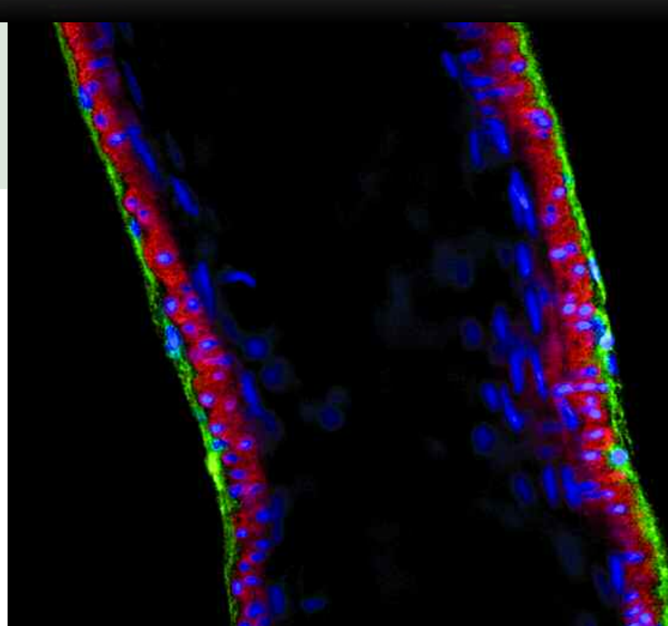


Figure 6:

CK-2018571 relaxes skinned, thiophosphorylated caudal artery rings from Sprague Dawley rats. Force development is expressed as a percentage of the initial force (n=8-12, mean +/- sem).



Conclusions

1. CK-2018571 selectively inhibits the ATPase activity of smooth muscle myosin as compared to other myosin II isoforms (non-muscle myosin, cardiac and skeletal muscle myosins).
2. CK-2018571 inhibits smooth muscle myosin in a weak actin-binding state, consistent with its ability to relax smooth muscle tissue *in vitro*.
3. CK2018571 inhibits the chemical cleavage of ATP by smooth muscle myosin, a mechanism distinct from previously identified myosin inhibitors such as blebbistatin and BTS.
4. CK-2018571 inhibits calcium-induced force development in skinned caudal artery and relaxes skinned rings activated by thiophosphorylation, consistent with relaxation occurring as a consequence of the direct inhibition of smooth muscle myosin.

References

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