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Forces measured with micro-fabricated cantilevers during actomyosin interactions produced by filaments containing different myosin isoforms and loop 1 structures

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ABSTRACT

Background: There is evidence that the actin-activated ATP kinetics and the mechanical work produced by muscle myosin molecules are regulated by two surface loops, located near the ATP binding pocket (loop 1), and in a region that interfaces with actin (loop 2). These loops regulate force and velocity of contraction, and have been investigated mostly in single molecules. There is a lack of information of the work produced by myosin molecules ordered in filaments and working cooperatively, which is the actual muscle environment.
Methods: We use micro-fabricated cantilevers to measure forces produced by myosin filaments isolated from mollusk muscles, skeletal muscles, and smooth muscles containing variations in the structure of loop 1 (tonic and phasic myosins). We complemented the experiments with in-vitro assays to measure the velocity of actin motility.
Results: Smooth muscle myosin filaments produced more force than skeletal and mollusk myosin filaments when normalized per filament overlap. Skeletal muscle myosin propelled actin filaments in a higher sliding velocity than smooth muscle myosin. The values for force and velocity were consistent with previous studies using myosin molecules, and suggest a close correlation with the myosin isoform and structure of surface loop 1.
General significance: The technique using micro-fabricated cantilevers to measure force of filaments allows for the investigation of the relation between myosin structure and contractility, allowing experiments to be conducted with an array of different myosin isoforms. Using the technique we observed that the work produced by myosin molecules is regulated by amino-acid sequences aligned in specific loops.

1. Introduction

The muscle molecular motor myosin II uses energy derived from ATP hydrolysis to perform mechanical work and move actin filaments, thereby generating force [55]. Although the molecular mechanism behind energy transduction from ATP hydrolysis into mechanical work is still under investigation, there is evidence that the actin-activated ATP kinetics and ADP release are fine-tuned in the myosin molecule by two surface loops. These loops are present in all myosins [55], and are situated close to the nucleotide pocket and the actin binding site, respectively [14,44], aligning residues 204–216 (loop 1) and 627–646 (loop 2) in the chicken myosin heavy chain (MHC) sequence [48,49]. The length and sequence of these loops vary considerably among myosins [64], suggesting that they have important regulatory functions.

Experiments with Dictyostelium myosin [27,42], phasic and tonic isoforms of smooth muscle myosin [2,25,26,59], and artificial myosin constructs [6,32,33,50] suggest that loop 1 is responsible for modulating ADP release according to specific mechanical demands. Experiments with Dictyostelium myosin [28,60] and artificial constructs [21,22,43] suggest that loop 2 is responsible for modulating actin-activated ATPase activity. While cleavage of loop 1 does not affect ATPase activity, cleavage of loop 2 does not affect ADP release, conferring a highly specific role for their structures [3]. In the case of smooth muscle myosins, the difference in loop structures and their relation with motor activity is well defined: they contain two isoforms with a natural mRNA splicing at the amino-terminus, differing by the presence (+) or absence (−) of the 7 amino acid insert (QGPSFAY). The insert is present in the rapidly contracting "phasic" muscle myosin, while it is not present in the slowly contracting "tonic" muscle myosin. Accordingly, the phasic myosin

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induces a higher actin motility velocity than the tonic myosin isoform [13,32,33,59], a likely result of a faster myosin power-stroke dictated by ADP release during ATP hydrolysis.

Significant information about the mechanics of contraction has been derived from studies performed using the laser trap technique, which allows measurements of force in single myosin molecules, e.g. [12,23,32,33]. Although the laser trap is a powerful tool for scientists to exploit the mechanisms of the myosin power stroke, myosin molecules in skeletal and smooth muscles are assembled in filaments. The cooperative action of myosin molecules is amplified, and several actin filaments slide to produce muscle contraction. Thus, the kinetics and mechanical characteristics measured in studies using single molecules and filaments may not be necessarily similar, and cooperativity among molecules may alter the forces and velocity produced by the filaments, i.e. each myosin molecule may influence and be influenced by an adjacent molecule. While interpretation of laser trap studies assumes that the myosin power stroke is similar to that of naturally oriented myosin molecules in the thick filaments, e.g. [51], the error associated with the orientation of myosin can amount to a factor of two [20,56]. Furthermore, it is assumed that the myosin power stroke occurs with equal probability at all positions along the actin filament, but forces generated by the laser trap may induce distortion of the myosin molecules, resulting in non-equal probability. Unfortunately, experiments with myosin filaments are not common, as they are technically challenging [36] and depend on simultaneous visualization of the myosin and actin filaments during experiments [24]. Furthermore, polymerization of myosin filaments with a specific length, thickness and number of molecules is not trivial, and may lead to substantial alterations in myosin–actin interactions.

Lately, a great attention has been given to the use of microelectromechanical systems (MEMS) or micro-fabricated devices for investigating biological processes [11,24], including the development of neuromuscular junctions [17,18], spinal reflex arc [68], muscle tissue engineering and myotube development [8–10]. The latest is particularly important in the context of this study; cantilever-based MEMS can be used for the investigation of contractile stress produced by cultured skeletal muscles [67], opening several possibilities for the study of muscle contraction in general.

In the current study, we present experiments conducted with a newly developed system that uses micro-fabricated cantilevers to measure forces produced by myosin filaments when interacting with actin. The filaments were isolated from mollusks and rabbit psoas muscles, or were polymerized with myosin molecules from skeletal and smooth muscles containing variations in the structure of loop 1. We complemented the experiments with in-vitro motility assays for measurements of myosin-propelled actin velocity. Our results showed that force produced by smooth muscle myosin is greater than skeletal muscle myosin, but they caused actin filaments to slide at a slower velocity, with values well within ranges reported by studies using single myosin molecules.

2. Methods

2.1. Protein preparation and polymerization of myosin

Five different myosin filaments were used in this study: (i) native thick filaments isolated from the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* (mussel), (ii) native thick filaments isolated from the psoas muscle from the rabbit, (iii) filaments polymerized from chicken breast skeletal muscle myosin [54], (iv) filaments polymerized from turkey gizzard phasic smooth muscle myosin, and (v) filaments polymerized from a mixture (50–50%) of phasic and tonic smooth muscle myosin purified from turkey gizzard and pig stomach fundus [54]. The two types of smooth muscle myosin were used because they differ in their natural mRNA splicing at the amino-terminus, with a presence (+) or absence (−) of the 7 amino acid insert at the junction of loop 1 (QGPSFAY). These differences in loop 1 correspond to varying contractile properties of smooth muscles, including difference in the velocity of contraction and ATPase kinetics [34]. The myosins were thiophosphorylated in 20 mM KCl buffer [58].

Native mussel thick filaments were isolated following standard procedures [24,53]. Pairs of anterior byssus retractor muscles were extracted from fresh mussels and placed in thick filament buffer solution (10 mM PIPES (pH 7.0), 10 mM $MgCl_2$, 2 mM EGTA, 10 mM ATP, 2 mM DTT). Five animals per preparation were used in this study. The muscles were diced into thin strips and homogenized on ice (SNMX 1092, Omni Inc) 3× for 7 s, with 1 min intervals. The homogenate was mixed with equal volume of the thick filament buffer solution containing 0.1% triton X-100, and left on ice for 15 min. The homogenate was precipitated in a centrifuge (5804R, Eppendorf) at 700 ×*g* for 5 min and the pellet was discarded. The supernatant was further centrifuged at 4500 ×*g* for 40 min, and the sediment was suspended in the thick filament buffer. These centrifugation steps were repeated, and thick filaments were re-suspended in a buffer solution in the absence of ATP.

Native thick filaments from the psoas muscle were isolated following procedures described by Trinick [57]. Briefly, two stripes of muscle (~3 mm in diameter, ~5 cm long) stored at −20 °C in Rigor solution mixed with glycerol were defrosted at 4 °C in Rigor solution. The muscles were left in a relaxing solution (100 mM KCl, 10 mM PIPES (pH 7.0), 10 mM $MgCl_2$, 2 mM EGTA, 10 mM ATP, 2 mM DTT) for 1 h, before being diced into thin strips and homogenized on ice (SNMX 1092, Omni Inc) 2× for 30 s, with 1 min intervals. The homogenate was centrifuged (5804R, Eppendorf) at 4500 ×*g* for 30 min to remove undamaged myofibrils and other contaminants.

For polymerization of skeletal and smooth muscle myosin filaments, the protocol followed previous studies [47] with minor modifications using solutions with high KCl concentration (10 mM Immidazol-HCl (pH = 6.8), 500 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT) and low KCl concentration (10 mM Immidazol-HCl (pH = 6.8), 80 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT). Small amounts (3 μl) of stock skeletal muscle myosin solution (22 mg/ml) were placed in the high KCl solution to obtain 100 μl of myosin at 0.7 mg/ml. KCl concentration was slowly brought down to 120 mM by diluting it at constant speed with the low KCl solution (dilution speed: 0.01 ml/min, dilution time: ~2 h), using a syringe pump (Pump 33, Harvard Apparatus, USA). Polymerization of smooth muscle myosin followed the same protocol, except that the pH which was adjusted to 6.2 to form long and wide filaments [47]. All procedures were performed at room temperature. The length of thick filaments was measured during experiments using images analyzed with ImageJ software (length measurement tool), and confirmed with electron microscopy analysis (see below).

Unregulated actin was purified from chicken pectoralis acetone powder following the protocol developed by Pardee and Spudich [45]. Actin filaments were prepared following standard procedures [46], or were kindly provided by Dr. Mike Regnier (University of Washington, USA). The filaments were labeled with Alexa-488-phalloidin fluorescence dye (absorption/emission peaks at 488–520 nm). α-actinin (A9776, Sigma) was dialyzed against AB buffer (25 mM imidazole-HCl (pH 7.4), 25 mM KC1, 4 mM $MgC1_2$, 1 mM EGTA, 1 mM DTT) and was used in the experiments to strengthen the attachment of actin to micro-fabricated cantilevers, which were used to measure forces generated during filament interactions (see below).

2.2. SDS–PAGE and Western blotting of tonic and phasic myosin

Equal amounts of myosin were loaded onto SDS–PAGE, using 4–20% TGX gradient gel (Bio-Rad, Hercules, CA). The protein concentration was estimated by a standard Bradford assay and 0.13 μg of myosin were loaded in each well. Proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules,