A single-fiber in vitro motility assay. In vitro sliding velocity of F-actin vs. unloaded shortening velocity in skinned muscle fibers

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Abstract

We describe an approach that allows us to form a micro in vitro motility assay with as little myosin as can be retrieved from a short (~10 mm) segment of a single skinned skeletal muscle fiber (diameter some 100 μm). Myosin is directly extracted from the single fiber segment by a high ionic strength solution in the presence of MgATP, and the extracted myosin is immediately applied to a miniaturized flow cell that has been pretreated with BSA. The observed sliding velocities of fluorescently labeled F-actin are essentially identical with those reported in the literature. Since at the single fiber level most muscle fibers contain only a single myosin heavy chain isoform this approach allows us to determine without additional purification steps, the sliding velocity driven by myosins with different heavy chain isoforms. In addition, this approach can be used to directly correlate under identical experimental conditions unloaded shortening velocity measured in segments of skinned muscle fibers with the in vitro sliding velocity of fluorescently labeled F-actin by extraction of myosin from the same skinned fibers. Such direct correlation was performed with different myosin heavy chain isoforms as well as at different temperatures and ionic strengths. Under all conditions studied, unloaded shortening velocity was 4- to 8-fold faster than sliding velocity in the motility assay even at high temperature (22°C) and ionic strengths >50 mM. This suggests that sliding velocity in the motility assay is limited by additional factors beyond those thought to limit velocity of unloaded shortening in muscle fibers. One such factor might be unspecific ionic interactions between F-actin and the substrate in the motility assay resulting in somewhat higher sensitivity for ionic strength of sliding velocity in the motility assay. This might become of special relevance when using in vitro sliding velocity in assessing functional consequences of mutations involving charged residues of actin or myosin.

Abbreviations: BSA: bovine serum albumin; CPK: creatine phosphokinase; CrP: creatine phosphate; MHC: myosin heavy chain; SDS-PAGE: sodium dodecyl polyacrylamide gel electrophoresis; \( v_f \): in vitro sliding velocity; \( v_u \): unloaded shortening velocity.

Introduction

It is generally accepted that muscle contraction occurs when the thick myosin filaments slide past the thin actin containing filaments. This process is thought to be driven by a cyclic interaction of the heads of the myosin molecules with the actin filaments as ATP is hydrolysed. The details, however, as to how hydrolysis of ATP is coupled to force production and movement is still unclear. To develop an understanding of the molecular processes driving muscle contraction, in vitro motility assays have been developed (e.g., Sheetz and Spudich, 1983; Sheetz et al., 1984; Yanagida et al., 1984; Kron and Spudich, 1986; Kron et al., 1991; Harada et al., 1990; Sellers et al., 1993) to bridge the gap between biochemical studies in solution and physiological studies on intact or skinned muscle fibers. Biochemical studies in solution suffer from the loss of the three-dimensional arrangement of the myofilaments such that neither force-generation nor movement, the really relevant products of ATP-hydrolysis in muscle, can occur any more. Physiological studies on intact or skinned fibers, on the other hand, are complicated by components other than the actomyosin system, e.g., structures like the cytoskeleton or diffusion barriers like membrane systems. Furthermore, analysis of contractile function by recombinant proteins in fibers is so far limited to those proteins that are easily exchanged into the contractile system, e.g. troponin and its subunits or regulatory light chains. Finally, parameters observed in fiber studies represent the response of a large population of actomyosin interactions which makes unambiguous interpretation of the observed parameters in terms of function at the level of the single molecule difficult. In the in vitro motility assays, although the three-dimensional arrangement of the myofilaments is also lost, movement...
of actin filaments and even force measurements are still possible (cf. Sheetz and Spudich, 1983; Yanagida et al., 1984; Kron and Spudich, 1986; Kishino and Yanagida, 1988; Kron et al., 1991; Ishijima et al., 1991; Sellers et al., 1993). Advantages of in vitro motility assays over fiber studies are the much reduced complexity (e.g., no effects from cytoskeletal structures), the possibility to easily examine mutant proteins (Sutoh et al., 1991; Sutoh, 1993; Cuda et al., 1993, 1997; Ruppel et al., 1994; Uyeda et al., 1994, 1996; Anson et al., 1996), and the possibility to reduce the number of interacting molecules to individual molecules (Finer et al., 1994; Ishijima et al., 1994; Molloy et al., 1995). As a consequence, in vitro motility assays become more and more important for developing a detailed understanding of the molecular processes driving muscle contraction and cell motility.

To establish such central relevance, interpretation of the parameters observed in the in vitro motility assays has to be investigated and verified. For instance, interpretation of the sliding velocity observed in such in vitro motility assays is generally based on the assumption that the in vitro sliding velocity (v_f) is the equivalent of the maximum unloaded shortening velocity (v_u) observed in muscle fibers (e.g. Sheetz and Spudich, 1983; Sheetz et al., 1984). As a consequence, it has been widely assumed that, at the molecular level, v_f is limited by the same mechanism(s) as v_u, i.e., v_f is usually interpreted in terms of the mechanism that was proposed by A.F. Huxley (1957) to limit v_u. By comparing v_f-data recorded under various experimental conditions with v_u data reported in the literature Homsher et al. (1992, 1993) found substantial differences between v_f and v_u at low temperature and low ionic strength. At temperatures ≥20°C and ionic strengths >40 mM Homsher et al. (1992, 1993), however, concluded that there is excellent agreement between their v_f data and v_u data reported in the literature. Two problems, however, remained in this comparison; (i) v_f data were obtained at different experimental conditions (e.g., ionic and temperature conditions) than the v_u data quoted from the literature, and (ii) differences in fiber type and thus in myosin heavy chain isoforms made direct comparison difficult.

In order to test this central assumption in a most direct way, we developed a ‘single-fiber in vitro motility assay’ that allows direct comparison of v_u with v_f data recorded from the same myosin population under identical ionic and temperature conditions. With this modified in vitro motility assay, myosin extracted from a segment of a single skinned fiber is sufficient to generate a functional sliding assay and to identify the myosin heavy chain isoforms by SDS-PAGE. Thus, we can compare sliding velocity and unloaded fiber shortening velocity (i) from the same fiber segment and (ii) under identical experimental (ionic, temperature) conditions. In addition, extraction of myosin from single fibers allows us to compare in vitro sliding velocity and unloaded shortening velocity of skinned fibers for various MHC-isoforms since in many single fiber segments only a single MHC-isoform is expressed even in muscle of mixed fiber type (Aigner et al., 1993; Larsson and Moss, 1993; Sant’ana-Pereira et al., 1995; Harridge et al., 1996). Thus, our single-fiber in vitro motility assay allows to study sliding velocity of defined myosin heavy chain isoforms without the need of selective binding of MHC-isoforms to antibodies directed against specific (and different) sequences of the MHC. As a consequence, differences in sliding velocity that might be introduced by binding of the antibody to different areas of the myosin molecule (Winkelman et al., 1995) can be avoided.

With this approach we find that (i) for the range of conditions studied (10°C, 22°C, low and high ionic strength) in vitro sliding velocities of Rh–phalloidin labelled F-actin filaments in our motility assay are essentially identical with the data reported in the literature, and (ii) that the observed sliding velocities are 4- to 8-fold slower than the velocity of unloaded fiber shortening (v_u) recorded from the same fibers under the same ionic and temperature conditions. Part of this work has previously been reported (Thedinga and Brenner, 1995; Thedinga et al., 1996).

Materials and methods

Extraction of myosin. Myosin was extracted from rabbit soleus and psoas muscle, as well as from human soleus muscle. The human muscle tissue was material removed in the course of reconstruction surgery. From all three muscle tissues, single fibers were isolated from fiber bundles according to Yu and Brenner (1989). The skinning procedure was previously described in detail (Kraft et al., 1995b). For the present study, skinned fiber segments were isolated from bundles that were frozen in liquid propane and kept in liquid nitrogen according to the method of Kraft et al. (1995c). Myosin was extracted from these isolated segments of single fibers using a method modified from Hasselbach and Schneider (1951). The extraction buffer contained 500 mM NaCl, 10 mM HEPES, 5 mM MgCl2 and 2.5 mM ATP. In preparation for extraction of myosin, the segment of a single muscle fiber (approximately 10 mm in length, diameter about 100 μm) was wrapped around the tip of a glass pipette that was pulled from a capillary (4", 1.2 mm; World Precision Instruments Inc. Sarasota, FL, USA). The tip of the capillary, about 0.1 mm in diameter, was closed by melting before wrapping around the fiber. For extraction of myosin, the tip of the pipette with the fiber was placed into 5 μl of the extraction buffer held in an Eppendorf cup and kept at 4°C. After 30 min, the glass pipette with the extracted fiber was removed and the extraction solution was directly used for experiments.

For extraction of myosin from human soleus muscle the fiber segments were split longitudinally prior to incubation in the extraction buffer. Without such