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Phalloidin perturbs the interaction of human non-muscle myosin isoforms 2A and 2C1 with F-actin

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ABSTRACT

Phalloidin and fluorescently labeled phalloidin analogs are established reagents to stabilize and mark actin filaments for the investigation of acto-myosin interactions. In the present study, we employed transient and steady-state kinetic measurements as well as in vitro motility assays to show that phalloidin perturbs the productive interaction of human non-muscle myosin-2A and -2C1 with filamentous actin. Phalloidin binding to F-actin results in faster dissociation of the complex formed with non-muscle myosin-2A and -2C1, reduced actin-activated ATP turnover, and slower velocity of actin filaments in the in vitro motility assay. In contrast, phalloidin binding to F-actin does not affect the interaction with human non-muscle myosin isoform 2B and *Dictyostelium* myosin-2 and myosin-5b.
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1. Introduction

Actin is one of the most abundant proteins in eukaryotic cells and exists in a tightly controlled dynamic equilibrium between monomeric, globular G-actin and polymeric, filamentous F-actin. It plays a key role in a number of events in biological motility, including cell division and migration, muscle contraction, cytokinesis and intracellular transport [1].

Actin filament stability can be influenced by a change in the state of the bound nucleotide or the interaction of the filament with various actin binding proteins or drugs [2–4]. Phalloidin, a bicyclic heptapeptide toxin of the mushroom *Amanita phalloides*, binds to actin with high affinity at a 1:1 stoichiometric concentration [5,6], promotes actin polymerization [7], and stabilizes the filaments by reducing the subunit dissociation rate constants to near zero at both ends of the polymer [8,9]. Due to these unique properties the reagent and its fluorescent derivatives are widely used for localizing actin in living cells [10] and to stabilize and visualize individual actin filaments in commonly used in vitro motility assays [11,12]. Several studies report that phalloidin affects various properties of filamentous actin, e.g., bending and torsional flexibility [13,14], enhances both force and ATPase activity in skinned striated muscle fibers [15,16], and slows down rabbit skeletal muscle myosin-2 based actin sliding in an in vitro motility assay [13,17]. However, numerous investigations of acto-myosin function are based on the use of phalloidin-decorated actin filaments and the often-held view is that they behave in their interaction with myosin identical to native actin filaments [18].

Here, we present evidence that the effects of phalloidin-stabilization can greatly vary even between closely related myosins. The function of human non-muscle myosin isoforms 2A and 2C1 is perturbed by phalloidin-stabilization of F-actin. In contrast, the third human non-muscle myosin isoform 2B and class-2 and class-5 isoforms from *Dictyostelium* are not affected by phalloidin binding to F-actin.

2. Materials and methods

2.1. Plasmid construction

Expression cassettes were generated encoding the motor domain region of human non-muscle myosin-2A (residues 1–775), -2B (residues 1–782), or -2C (residues 1–807), each fused with two *Dictyostelium* α-actinin repeats, and an octahistidin-tag. In the case of non-muscle myosin-2A and -2B, the expression cassettes were used in the BP recombination reaction according to the manufacturer (Invitrogen) with the vector pDONR201. The entry-vector was used in the LR reaction according to the manufacturer's instruction with the vector pDEST8. In the case of non-muscle myosin-2C, we generated an expression vector for the production of the motor domain of the splice variant 2C0. The expres-

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sion cassette was synthesized by Eurofins-MWG-Operon (Ebersberg, Germany) with optimized codon usage for the protein production in the baculovirus/Sf9-system and subcloned in the vector pFastBac1 (Invitrogen). The 8 amino acid extension of loop-1 in isoform 2C1 was integrated via a PCR approach. Expression vectors for *Dictyostelium* myosin-2 and myosin-5b motor domains, encoding amino acids 1–765 and 1–839, followed by two *Dictyostelium* α-actinin repeats and an octahistidin-tag, were generated as described previously [19,20]. All plasmids were verified by sequencing.

2.2. Protein preparation

Human non-muscle myosin-2A, -2B and -2C1 motor domain constructs were produced in *Sf9* insect cells. *Dictyostelium* myosin-2 and myosin-5b motor domain constructs were produced in *Dictyostelium discoideum* [21]. Proteins were purified by Ni^{2+}-chelate-affinity chromatography and gel filtration chromatography. Rabbit skeletal muscle α-actin was purified by the method of Lehrer and Kerwar [22]. Briefly, G-actin was obtained in a buffer containing 5 mM Tris–HCl, pH 7.5, 0.2 mM $CaCl_2$, 1 mM NaN_3 and 0.1 mM ATP. Polymerization was performed by adding 100 mM KCl and 2 mM $MgCl_2$ and incubation at 4 °C over night. The solution was centrifuged at 70 000×*g* at 4 °C for 1 h and the resulting F-actin pellets were homogenized in actin buffer (5 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$ and 0.04% NaN_3). The purity of the protein was confirmed by SDS–polyacrylamide gel electrophoresis (Supplementary Fig. 1). To prepare phalloidin-stabilized actin filaments a stock solution of 100 μM F-actin and 100 μM phalloidin (Sigma) was incubated in actin buffer on ice over night. Actin filaments were fluorescently labeled with N-hydroxysuccinimide (NHS)-rhodamine. The labeling was performed using a NHS-Rhodamine Protein Labeling Kit (Pierce) according to the manufacturer's protocol. To label F-actin with TRITC-phalloidin (Sigma) the reagents were mixed vigorously in a 1:1.5 M ratio in AB-buffer (25 mM Imidazole, pH 7.4, 25 mM KCl, 4 mM $MgCl_2$ and 1 mM EGTA) and incubated on ice over night.

2.3. ATPase assay

Comparative steady-state ATPase activities of non-muscle myosin-2 with phalloidin-free or phalloidin-stabilized F-actin were measured at 25 °C using the NADH-coupled assay according to Furch et al. [23] in a buffer containing 25 mM HEPES, 4 mM $MgCl_2$ and 0.5 mM DTT. The myosin concentration was 1 μM. The reaction was initiated by adding 1 mM ATP to the mixture. NADH oxidation was followed using the change in absorption at 340 nm in a Beckman DU-800 spectrophotometer.

2.4. Flash-photolysis experiments

Transient kinetic measurements were performed at 35 °C using a flash photolysis system as described by Weiss et al. [25]. One micromolar myosin was mixed with 0.5 mM caged-ATP (cATP) and either 5 μM phalloidin-free or phalloidin-decorated F-actin in a buffer containing 50 mM KP_i, pH 7.4, 400 mM KCl, 5 mM $MgCl_2$ and 10 mM DTT. A flash of UV light was used to excite cATP. The amount of released ATP was calculated from the increase of absorbance at 405 nm due to the formation of the excited aci-nitro state. The interaction of myosin with F-actin and ATP was followed by observing changes in light scattering.

2.5. In vitro motility assay

Standard in vitro motility assays were performed using an Olympus IX81 (Olympus) inverted fluorescence microscope as described previously [19,24]. Assays were performed at 30 °C in AB-buffer with 10 mM DTT and 4 mM ATP. The influence of phalloidin on the productive interaction of non-muscle myosin-2 isoforms with F-actin was analyzed by measuring the sliding velocity of either directly labeled rhodamine F-actin or TRITC-phalloidin labeled F-actin on myosin-coated surfaces. For each experiment, 0.2 mg/ml anti-His antibody immobilized myosin and 20 nM labeled F-actin were used. The movement of at least 50 filaments was recorded. Automated actin filament tracking was performed with the program DiaTrack 3.01 (Semasopht, Switzerland) and the average sliding velocity was determined by the analysis of the Gaussian distribution with Origin 7.0 (Originlab, USA).

3. Results

We chose a multifaceted approach to analyze the effect of phalloidin on the interaction of human non-muscle myosin isoforms 2A, 2B and 2C1 with F-actin. First, we investigated the ATP-induced dissociation of the acto-myosin complex in the absence and presence of phalloidin by flash photolysis (Fig. 1). The reaction was monitored by the large change in the light scattering signal that follows the light induced release of ATP. The rate constant k_{obs} for the ATP-induced dissociation of the acto-myosin complex was obtained by fitting a single exponential to the data. The experiment was performed with flashes releasing ATP concentrations in the range from 5 to 20 μM and the obtained rate constants were linearly dependent on the concentration of nucleotide (data not shown). The apparent second-order rate constants K_1k_{+2} for ATP binding to the acto-myosin complex were determined from the slopes of the straight lines fitted to the data. In the presence of phalloidin K_1k_{+2} values for non-muscle myosin-2A and non-muscle myosin-2C1 were reduced from ~0.7 to ~0.3 $\mu M^{-1}\,s^{-1}$. No change in K_1k_{+2} was observed with non-muscle myosin-2B (Table 1).

The final increase of the light scattering signal due to the rebinding of myosin to the actin filament after complete hydrolysis of the released amount of ATP is described best by a sigmoidal curve defining the rate constant k_{ATPase}, corresponding to the steady-state ATPase rate of myosin at the given F-actin concentration (Fig. 1). Again, phalloidin decoration of actin affected the enzymatic activity of non-muscle myosin-2A and -2C1. In the presence of 5 μM phalloidin-stabilized F-actin the two isoforms exhibited a 3- and 5-fold slower ATPase activity while non-muscle myosin-2B displayed the same actin-activated ATPase rate in the absence and

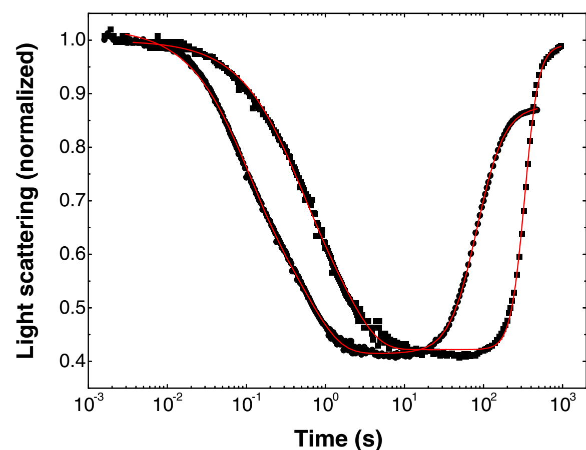

Fig. 1. Flash photolysis with acto-non-muscle myosin-2C1 (●) and phalloidin-stabilized acto-non-muscle myosin-2C1 (■) at 35 °C. The light-induced conversion of cATP into free ATP is followed by dissociation of the acto-myosin complex, ATP turn-over, and rebinding of myosin to F-actin. The three associated phases are monitored by changes in the intensity of the light scattering signal.

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