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Research article

Effects of cytoskeletal drugs on actin cortex elasticity

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ABSTRACT

Mechanical properties of cells are known to be influenced by the actin cytoskeleton. In this article, the action of drugs that interact with the actin cortex is investigated by tether extraction and rheology experiments using optical tweezers. The influences of Blebbistatin, Cytochalasin D and Jasplakinolide on the cell mechanical properties are evaluated. The results, in contradiction to current views for Jasplakinolide, show that all three drugs and treatments destabilize the actin cytoskeleton, decreasing the cell membrane tension. The cell membrane bending modulus increased when the actin cytoskeleton was disorganized by Cytochalasin D. This effect was not observed for Blebbistatin and Jasplakinolide. All drugs decreased by two-fold the cell viscoelastic moduli, but only Cytochalasin D was able to alter the actin network into a more fluid-like structure. The results can be interpreted as the interplay between the actin network and the distribution of myosins as actin cross-linkers in the cytoskeleton. This information may contribute to a better understanding of how the membrane and cytoskeleton are involved in cell mechanical properties, underlining the role that each one plays in these properties.

1. Introduction

The cell surface is composed by the plasma membrane and the cortical cytoskeleton underneath. These two structures form the membrane-cytoskeleton complex, an important regulator of several cellular processes, ranging from cell shape control and migration to cell division and differentiation [1–3]. The plasma membrane with its associated proteins separates the cytoplasm from the extracellular medium, participating in many cellular responses such as molecule recognition, signaling and cytokinesis [4,5]. The cortical cytoskeleton is a highly labile and cross-linked actomyosin network that gives support to the plasma membrane [2,6]. Actin polymerization dynamics and myosin contractility on actin fibers contribute to mechanotransduction, migration and embryogenesis [2,7,8]. The membrane-cytoskeleton complex is able to exert and react against forces owing to its mechanical properties. Different micromanipulation tools such as magnetic tweezers, atomic force microscopy, micropipette aspiration and optical tweezers have been applied to characterize cell mechanical properties [9].

Membrane tether extraction uses optical tweezers (OT) to create a membrane tube from the surface of cells [10–13] or other membranous structures [14–16]. By measuring both the force to produce the tether and the tether radius, the cell membrane tension (CMT) and the cell membrane bending modulus (CMBM) can be determined [10–12]. The CMT has been recently described as an important regulator of many cellular events [17–19].

Membrane tethers pulled away from cell surfaces contain filamentous actin (F-actin) [10,11]. The presence of F-actin inside tethers contributes to the occurrence of sawtooth-like peaks in tether force [20,21] and influences the CMT and the CMBM [10]. Indeed, fibroblasts treated with 5 μM of Cytochalasin D, a drug that disrupts the actin cytoskeleton, exhibit higher values for the CMBM [10]. This result might arise from a variety of factors, such as the effects of F-actin interaction with transmembrane or peripheral proteins, linkers or molecular motors [22]. It reinforces the need to better evaluate the effects of actin polymerization dynamics on cellular cortices.

Regarding cytoskeleton modeling, one of several proposals considers it as an active soft glassy material with storage and loss moduli varying according to cellular functions [23,24]. Rheology measurements of the cytoskeleton have shown universal dependence on the frequency of the applied mechanical stimulus and active response to

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stress, rendering the meshwork a more solid-like behavior for a constant stretch and a more liquid-like for a transient one [1,25–27]. Results for cytoskeletal storage and loss moduli show differences of up to two orders of magnitude [28–33]. In this context, a method based on finite element analysis [34] combined with OT has been recently applied under tightly controlled conditions to determine the viscoelastic properties of different cell types [35]. On the other hand, the use of various cytoskeleton-destabilizing drugs on a wide variety of cell types has also been investigated [36–43] and it represents a valuable pharmacological tool for evaluating the role of the cytoskeleton in many cellular processes. Effects of membrane composition (cholesterol) have also been investigated by optical tweezers techniques [12,44].

In the present work, we combine tether extraction, rheology and drug treatments with fluorescence microscopy observations, to investigate the influence of cytoskeleton integrity on the mechanical and viscoelastic properties of cells, mainly NIH3T3 fibroblasts. We use three different drugs: Cytochalasin D, Blebbistatin and Jasplakinolide. The first two are known to destabilize the actin cytoskeleton, whereas the third is believed to stabilize it. Surprisingly, we find that it also induces destabilization. OT are used to determine both the CMT and the CMBM, as well as the storage and loss moduli. Our aim is to collect a maximum amount of experimental evidence that may provide a basis for future detailed molecular biology elucidation of the membrane-cytoskeleton interaction.

We interpret our findings as resulting from the interplay between the actin network and the distribution of myosin cross-linkers in the cytoskeleton. This information may contribute to a better understanding of how the membrane-cytoskeleton complex is involved in cell mechanical properties.

2. Materials and methods

2.1. Cell culture and treatments

Swiss mouse embryo fibroblast NIH3T3 cells were cultured in DMEM-F12 medium supplemented with 1% L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Cells were maintained at 37 °C and 5% CO_2. 2×10^5 cells were plated on the day before the experiments in 18×18 mm glass coverslips pre-coated with poly-L-lysine, and placed on a 35 mm glass-bottom dish. In order to destabilize the actomyosin cytoskeleton, different drugs were used: 1.0, 2.5 and 5.0 μM of Cytochalasin D (CytoD); 2.5, 5.0 and 10 μM of Blebbistatin (BBI) and 10, 20 and 50 nM of Jasplakinolide (JPK). All drugs were purchased from Sigma-Aldrich (St. Louis, MO). NIH3T3 cells were treated for two hours prior to experiments with each drug condition. All drugs were kept in contact with cells throughout the experiments.

2.2. Fluorescence microscopy

NIH3T3 cells, previously treated with each drug condition, were subsequently fixed in PBS+4% paraformaldehyde for 15 min, treated with PBS+0.2% Triton-X100 for 5 min, blocked with PBS+5% BSA for 1 h at 37 °C and incubated for 1 h with Phalloidin-FITC (Sigma-Aldrich, St. Louis, MO) at 37 °C. Images of NIH3T3 cells in each condition were acquired using a Leica TCS-SP5 II confocal microscope (Leica Microsystems, Germany). Confocal fluorescence images were captured employing LAS AF 2.2.0 Software (Leica Microsystems, Germany). Quantification analysis of Phalloidin-FITC fluorescence was performed using the FibrilTool plug-in [45]. This plugin is an ImageJ (National Institutes of Health, Bethesda, MD) adapted software that determines the average orientation of a fiber array and provides a quantitative description of the fiber array anisotropy. The calculations are based on the concept of nematic tensors, used to describe liquid crystals. The anisotropy parameter value is maximum when all the fibers in the array point to the same direction and zero when they are randomly oriented.

In order to better understand how JPK was remodeling the actin cytoskeleton, NIH3T3, U2OS, MDCK and mouse embryonic fibroblast (MEF) cells were used. MEF and MDCK cells were cultivated similarly to NIH3T3 cells and as previously described. U2OS cells were cultivated in McCoy's 5 A media (Sigma-Aldrich) supplemented with 10% FBS (HyClone; Thermo Fisher Scientific), 2 mM L-glutamine and penicillin-streptomycin. All cell lines were transiently transfected with an actin-GFP construct (a gift from Clair Waterman), the day before the JPK treatments, using a Neon® electroporation system (Life Technologies, USA) and following the manufacturer's protocol. 10, 25 and 50 nM of JPK were added in each of these cell cultures. The GFP-actin transient transfection protocol was used, as opposed to phalloidin-FITC, because JPK is known to compete with phalloidin for the same binding sites in actin filaments [46]. After the 2 h treatments, cells were fixed for 15 min with PBS+4% paraformaldehyde, washed three times with PBS and then imaged using the same procedures described above.

2.3. Optical tweezers

The OT system has been previously described [11,35]. Briefly, it uses an infrared laser (Nd: YVO4 Osprey laser, λ=1064 nm) incident on the back focal plane of a PLAN APO 100×1.4 NA DIC H Nikon objective attached to an inverted Nikon Eclipse TE300 microscope (Nikon, Melville, NY). The OT were calibrated and the trap transverse stiffness per unit power at objective entrance was $k/P = (0.12 \pm 0.02)$ pN μm^{-1} mW^{-1}.

2.4. Tether extraction experiments

Tether extraction experiments were performed as described before [10–12]. Briefly, 2×10^5 NIH3T3 cells previously plated and treated with each drug condition were placed in the OT microscope. Uncoated polystyrene beads, of radius $a = (1.52 \pm 0.02)$ μm (Polysciences, Warrington, PA) were added and the OT were used to trap a bead and to press it against the membrane of a chosen cell for 5 s, producing bead attachment. The microscope stage (Prior Scientific, Rockland, MA) was then set to move with a controlled velocity of 1 μm/s. Images of the entire experiment were collected at a rate of 10 frames/second. Using the OT calibration and bead displacement, obtained by image analysis, the tether force was determined. All OT experiments were performed in optimal culture conditions (37 °C and 5% CO_2).

The CMT (σ_{eff}) is given by

$$\sigma_{eff} = \frac{F_0}{4\pi R}, \tag{1}$$

and the CMBM (κ_{eff}) is

$$\kappa_{eff} = \frac{F_0 R}{2\pi}, \tag{2}$$

where F_0 is the tether force and R is the tether radius [10,11,47–49], obtained as equilibrium values in tether extraction experiments with cells. They are defined as effective values, since the free energy used to derive Eqs. (1) and (2) strictly applies only to membrane tethers with no cytoskeleton content. Data analysis and force calculations were performed using Kaleidagraph software (Synergy Software, Essex Junction, VT).

2.5. Measurements of tether radius extracted from cells

Immediately after tether extraction experiments, the beads used to extract the tethers were attached to the coverslip. The samples were then fixed and prepared for scanning electron microscopy following the same protocol used before [10–12]. After image acquisition and