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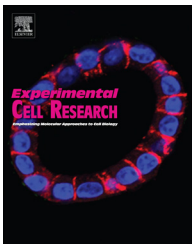

Research Article

Microtubules mediate changes in membrane cortical elasticity during contractile activation

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ABSTRACT

The mechanical properties of living cells are highly regulated by remodeling dynamics of the cytoarchitecture, and are linked to a wide variety of physiological and pathological processes. Microtubules (MT) and actomyosin contractility are both involved in regulating focal adhesion (FA) size and cortical elasticity in living cells. Although several studies have examined the effects of MT depolymerization or actomyosin activation on biological processes, very few have investigated the influence of both on the mechanical properties, FA assembly, and spreading of fibroblast cells. Here, we examine how activation of both processes modulates cortical elasticity as a function of time. Enhancement of contractility (calyculin A treatment) or the depolymerization of MTs (nocodazole treatment) individually caused a time-dependent increase in FA size, decrease in cell height and an increase in cortical elasticity. Surprisingly, sequentially stimulating both processes led to a decrease in cortical elasticity, loss of intact FAs and a concomitant increase in cell height. Our results demonstrate that loss of MTs disables the ability of fibroblast cells to maintain increased contractility and cortical elasticity upon activation of myosin-II. We speculate that in the absence of an intact MT network, a large amount of contractile tension is transmitted directly to FA sites resulting in their disassembly. This implies that tension-mediated FA growth may have an upper bound, beyond which disassembly takes place. The interplay between cytoskeletal remodeling and actomyosin contractility modulates FA size and cell height, leading to dynamic time-dependent changes in the cortical elasticity of fibroblast cells.

Introduction

The cytoskeleton is an interconnected structure composed primarily of actin, microtubules (MTs) and intermediate filaments, which is required for a vast number of cellular processes [1–6]. These filamentous networks play crucial roles in dynamic changes in cell shape, migration, and adhesion. Importantly, changes in the composition and structure of the cytoskeleton during physiological and pathological processes often correlate with distinct changes in the mechanical properties of the cell [1,4,7]. In turn, the mechanical properties of the microenvironment also regulate many of these processes [1,4,7]. Atomic force microscopy (AFM) has emerged as one of several tools employed to investigate and quantify the mechanical properties of cells and their responses to

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mechanical stimuli [7,8]. Using this method, dynamic changes in the cell's elastic properties can be quantified during biological processes [1,7]. In addition, AFM has also become an attractive tool for examining how cells respond to local nanoscale forces [7,9].

Selective removal of specific molecules, through genetic or biochemical means, is often employed to disrupt the cytoskeleton, in order to systematically study the effects on cortical elasticity. Depolymerization of intact actin filaments (cytochalasin D), or inhibition of myosin-II activity (blebbistatin), has been shown to cause a decrease in cortical elasticity [10–14]. Conversely, increasing myosin-II contractility with calyculin A (CalA) results in an increase in cortical elasticity [15,16]. Moreover, increased myosin-II contraction also results in active assembly of FAs [14,17–20], leading to increased cellular traction [15,16]. MTs can signal to the actomyosin network through Rho family GTPases, which are essential elements in the regulation of the actomyosin cytoskeleton [21,22]. The disruption of MTs leads to an increase in actomyosin contractility, traction force magnitude, and cortical elasticity [5,23–25]. Therefore, there are two possible pathways in which cellular cortical elasticity can be increased – through MT depolymerization or actomyosin activation. CalA and nocodazole are two such agents that can either depolymerize MTs or enhance actomyosin contractility, respectively [12,13,15,26,27]. CalA is a phosphatase inhibitor that causes myosin-II over-activation [14,20]. On the other hand, nocodazole treatment inhibits MT polymerization, leading to increased stress fiber formation and contraction over short timescales (less than 30 min) [23,24,28]. Previous studies have shown that either the activation of contractility, or the depolymerization of MTs, can cause an increase in cell spreading, changes in cellular morphology, and FA assembly [5,14,17,18]. However, up to date there is no systematic study involving activation of both pathways, individually and sequentially, on cortical elasticity, FA assembly, and spreading of NIH3T3 fibroblasts.

In this light, the objective of this study was to examine the effect of activating, an increase in contractility by sequentially increasing myosin-II activity and depolymerizing MTs. We show that a time-dependent increase in cortical elasticity can occur when myosin-II activation (CalA) or MT depolymerization (nocodazole) occurs individually. However, the sequential application of CalA followed by nocodazole did not elicit an additive result. Instead, the sequential use of these two drugs led to a decrease in cortical elasticity. This loss of stiffness occurred with a concomitant decrease in FA size and an increase in cell height. Clearly, MT inhibition disables the ability of fibroblast cells to maintain their contractility and cortical elasticity upon activation of myosin-II. These results illuminate the complex interplay between cytoskeletal and FA remodeling dynamics that ultimately control the mechanical properties of these cells. It should be noted that the observed changes in cortical elasticity were highly time dependent. Understanding how cortical elasticity is regulated will provide insight into the mechanisms that link signaling pathways to remodeling dynamics in the cytoarchitecture [29–31].

Materials and methods

Cell culture, reagents and transfections

NIH3T3 fibroblast cells were maintained in a standard incubator in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% streptomycin/penicillin (Hyclone Laboratories). Cells were cultured on 0.1% gelatine-coated 35 mm plastic dishes at a density of $\sim 10^5$ cells/cm^2. All drugs (Sigma) were stored as stock solutions in DMSO. Calyculin A (CalA) and nocodazole were used at final concentrations of 1 nM and 10 μM, respectively. These exact concentrations and time durations were used in order to draw insightful comparisons with previous studies [14,15,17,32–35]. Secondly, when these drugs were used individually, they allowed us to determine a practical experimental incubation time required to achieve a significant increase in cortical elasticity. In the case of CalA, higher concentrations (100 nM) are known to cause rapid cell rounding and detachment due to increased contractility [17,35]. Cell detachment and dramatic changes in shape make comparisons to relatively well-spread and adhered cells very difficult. Therefore, a lower concentration of CalA (1 nM) was chosen in order to maintain the general morphology of adherent fibroblast cells for the duration of our measurements. The chosen concentration of nocodazole (10 μM) is highly consistent with many studies on various cell types. In some cases, cells were transiently transfected with plasmids encoding for the pleckstrin homology (PH) domain of phospholipase C conjugated to EGFP (PH–PLCδ–EGFP) in order to label the cell membrane [34]. Transfections were performed using Lipofectamine 2000 (Invitrogen) and 1 μg DNA according to manufacturer's specifications.

Immunofluorescent staining

Immunofluorescence staining was carried out as previously described [34,36]. Briefly, cells were fixed with 3.5% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were then quenched in 0.15 M glycine for 25 min. Actin filaments were stained with Phalloidin Alexa Fluor 546, and nuclei were stained with DAPI (Invitrogen). Vinculin was labeled with a monoclonal mouse anti-vinculin antibody and a rabbit anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Following each step, cells were incubated in wash buffer (5% horse serum in PBS) for 15 min. For the MT stains, cells were fixed and permeabilized with methanol kept at −20 °C and immediately placed on ice for 3 min. MT filaments were labeled with an alpha-tubulin primary antibody produced in mouse and a rabbit anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Samples were mounted in Vectashield (Vector Labs) and a glass coverslip was placed on top. All imaging was carried out on an A1R laser scanning confocal microscope (LSCM) using a 60× water immersion objective (Nikon, Canada).

Atomic force microscopy (AFM)

An AFM (Nanowizard II, JPK Instruments) was used for all experiments with PNP-TR-50 cantilevers (Nanoworld) with spring constants of 69 ± 15 pN/nm. Two cells were randomly chosen from $n = 10$ plates and 5–15 force curves were measured on each cell over the nucleus, until 100 force curves in total were acquired. Force–displacement curves were recorded at 1 Hz with a velocity of 1 μm/s. The Sneddon model was employed to extract Young's modulus from a fit of the first 200 nm of force–indentation data in order to determine cortical elasticity [9,34,37]. The opening half angle of the AFM tip was 35° and the Poisson ratio was assumed to be 0.5. Data analysis was carried out using PUNIAS software