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.

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I N T R O D U C T I O N

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...
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 calycin A (CalA) results in an increase in cortical elasticity [15,16]. Moreover, increased myosin-II contraction also results in active assembly of FAs [14,17,18]. 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 ~10^5 cells/cm^2. All drugs (Sigma) were stored as stock solutions in DMSO. Calycin 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.
To assess the time-dependent response of cells to CalA and nocodazole, force curves were acquired after 5 and 30 min of exposure to either drug. At each time point force curves were acquired on two cells for no more than 2 min. Therefore, force curves were acquired between the 5–7 min and 30–32 min exposure time points for each drug. This process was then repeated on 10 dishes of cells. In some cases, cells were treated with CalA for 30 min, immediately followed by nocodazole for 5 min (or nocodazole (5 min) immediately followed by CalA (30 min)). In these scenarios, force curves were acquired on two cells between 35 and 37 min time points. This measurement was then repeated for 10 individual dishes of cells.

**Quantification of focal adhesion area, cell spreading area and cell height**

Image post-processing was carried out in ImageJ (http://rsb.info.nih.gov/ij/), where FA images were thresholded and segmented using an adaptive thresholding plugin (https://sites.google.com/site/qingzongteng/adaptivethreshold). Adaptive threshold overcomes the limitation of conventional thresholding when the feature intensities are not homogeneous. The three representative images in Fig. 4E are the most common focal adhesions sizes found in a cell. Here, we set both lower and upper bounds on FA size based on the distribution we observed, which were 0.5 μm² and 10 μm² respectively. LSCM images of cells stained for actin were thresholded as outlined above, and were used to determine cell area. Finally, LSCM orthogonal views of cells transiently expressing PH–PLCδ–EGFP were used to quantify cell height over the nucleus.

**Statistical analysis**

All presented values are the average±s.e.m., except for the AFM spring constants, which were presented as the average±s.d. To assess statistical significance ($P<0.05$), a two-sample t-test was performed.

**Results and discussion**

In this study, we investigated the role of microtubules in mediating cortical elasticity in mouse fibroblast cells during contractile activation. We stimulated the activation of two contractility pathways through the activation of myosin-II (CalA) or depolymerization of MTs (nocodazole). To assess how drug treatment duration affects the mechanical response of the cells (merization of MTs (nocodazole)). To assess how drug treatment pathways through the activation of myosin-II (CalA) or depolymerization. We stimulated the activation of two contractility 

30 min ($P=8.57 \times 10^{-22}$) and was consistent with a loss of intact MTs (Fig. 2). Importantly, this result was also observed when cells were exposed to nocodazole prior to CalA ($P=4.67 \times 10^{-3}$). Moreover, by imaging MTs following sequential incubation with both drugs, we observed a largely depolymerized MT network (Fig. 2). This counter-intuitive result, wherein cortical elasticity decreases upon sequential incubation with CalA and nocodazole, indicates that an increase in cortical elasticity, driven by myosin-II activation, can only persist in the presence of an intact MT network. However, the structural changes leading to the dynamics in cortical elasticity remain unclear. Therefore, we sought to examine changes in the actin cytoskeleton, FA structure, cell-spreading area and cell height as these are all known to influence cortical elasticity.

We first obtained LSCM images of actin filaments and vinculin for cells treated with 30 min of CalA, 5 min of nocodazole, or CalA and nocodazole (Fig. 3). Untreated fibroblasts appeared to be well spread with prominent FAs and actin fibers (Fig. 3A). CalA treated cells were well spread displaying actin filaments and more elongated FAs (Fig. 3B). After nocodazole treatment, both the actin and FAs remained intact (Fig. 3C). However, the combination of CalA and nocodazole resulted in a reduction of discrete FAs compared to untreated cells (Fig. 3D). Using these images, we were able to quantify FA and cell spreading area after each drug treatment (Fig. 3E and F). The individual or combined (regardless of the treatment order) drug treatments had dramatic effects on the size and distribution of FAs ($n=6–12$ cells, with ~150 FAs analyzed in total). FA morphology varied from the typical well-defined and elongated structure to more punctate assemblies (Fig. 3E). Upon individual treatments with CalA or nocodazole there was a significant increase in FA area ($P=5.60 \times 10^{-4}$ and $P=1.15 \times 10^{-3}$ respectively; Fig. 4B). In contrast, treatment with both drugs caused the FA area to decrease significantly ($P=1.02 \times 10^{-2}$; Fig. 4F). Moreover a significant decrease was also observed when nocodazole was employed prior to CalA ($P=1.06 \times 10^{-5}$). Taken together, the data suggests that the size of FAs correlates with the cortical elasticity of the cells.

Changes in cell height are commonly correlated with changes in local cortical stiffness [42–44], which is consistent with our results. Experiments were performed on cells expressing PH–PLCδ–EGFP [45] in order to provide a means of directly measuring cell height (Fig. 4A). LSCM images were obtained for all conditions ($n=9–14$ cells) and cell height was determined by measurement above the cell nucleus (stained with Hoescht 33342) from orthogonal maximum intensity projections (Fig. 4B). Individual treatment with CalA (30 min) and nocodazole (5 min) caused a complete depolymerization of the MT cytoskeleton was visible (Fig. 1B). The effect of nocodazole on the cortical elasticity of cells has been shown to vary widely and depends on cell type, substrate biochemistry, incubation time, or concentration [11,12,34,36,39–41]. Under the conditions of this study, the results reveal that the cortical elasticity of NIH3T3 cells undergoes a transient increase over short timescales (5 min), followed by a decrease over longer timescales (30 min).

Based on these observations, we hypothesized that sequential exposure to CalA for 30 min, followed by exposure to nocodazole for a further 5 min might lead to an additive response, and an even larger increase in cortical elasticity. However, this treatment protocol resulted in a significant decrease in cellular elasticity after 5 min of nocodazole exposure ($P=2.37 \times 10^{-40}$) (Fig. 2). This decrease in elasticity persisted, even after 30 min ($P=8.57 \times 10^{-22}$) and was consistent with a loss of intact MTs (Fig. 2).
significant decrease in height ($P=2.20 \times 10^{-2}$ and $P=4.63 \times 10^{-2}$, respectively; Fig. 4C). Treatment with both drugs (regardless of the treatment order) caused significant increases in cell height ($P=1.12 \times 10^{-3}$ and $P=7.68 \times 10^{-3}$; Fig. 3F). Changes in cell height are well known to inversely correlate with changes in cortical elasticity when measured with AFM [46–48]. Thinner portions of the cell (leading edges) are often observed to appear significantly stiffer than higher regions of the cell such as the...
nucleus [48]. Moreover, changes in cell height that occur during physiological changes (mitosis and apoptosis) have also been shown to exhibit corresponding changes in cellular elasticity [15,36,49]. Given that every cell is of different shapes and sizes, we chose to always measure cortical elasticity over the center of the nucleus. This provides a consistent region to sample from cell to cell. It is not immediately clear if the changes in cortical elasticity observed over the nucleus will be similar to off-nucleus regions. As we have previously shown, the cortical elasticity in off-nucleus regions can be significantly higher than the nuclear region due to lower cell height and a higher density of cytoskeletal filaments [48]. Off-nucleus regions also vary in size and shape, making generalizations and comparisons quite difficult. We speculate that the relative changes in cortical elasticity in off-nucleus regions of NIH 3T3 fibroblasts will be similar to on-nucleus regions. This speculation is motivated by the fact that the

Fig. 3 – Immunofluorescent images of NIH3T3 fibroblast cells following drug treatments. LSCM images of (A) untreated fibroblast cells (scale bar = 26 μm, applies to all) and after (B) CalA, (C) nocodazole or (D) both drugs. Cells were fixed and stained for vinculin (green), actin filaments (red) and DNA (blue). (E) LSCM images were post-processed in ImageJ, where FA and cell areas were segmented using available plugins. Three examples of how the segmenting process was carried out, showing the area for three different FAs. (F) FA area in response to each drug treatment. CalA or nocodazole alone caused an increase in FA area, however when used in combination (regardless of the exposure order) a decrease in FA area was observed.
relative increase in cell height over the nucleus also appears to be reflected in off-nucleus regions. However, extensive work would be required to systematically and carefully examine this question due to the complexities in the definition and accurate comparison of off-nucleus regions. Finally, the average cell spreading area displayed no significant differences compared to untreated cells due to a large degree of cell-to-cell variability in area (\( P > 0.1 \) in all cases; Fig. 4D).

Our results reveal an important time-dependent response to CalA and nocodazole as well as an important role MTs appear to play in mediating cortical elasticity during contractile activation. As both drugs act on equilibrium reaction pathways it is not surprising that there is a time-dependence to their action. Indeed, both drugs have been shown to display time and concentration dependent responses [50–52]. When CalA and nocodazole are used to treat cells separately, the results are straightforward and correspond to their known biochemical effects. CalA is a well-known inhibitor of MLC phosphatase (MLCPase), leading to an over abundance of phosphorylated MLC (MLCP) and increased myosin-II activity, downstream of Rho Kinase (ROCK) [35,52]. As CalA only inhibits the backwards reaction (MLCP dephosphorylation), there will be an associated time constant with its effect on cortical elasticity. Indeed, higher concentrations (100 nM) of CalA have been employed previously and result in more rapid cell rounding and detachment due to increased contractility [17,35]. Cell detachment and dramatic changes in cell shape make comparisons to relatively well-spread and adhered cells very difficult. Therefore, a low concentration of CalA (1 nM) was chosen in order to maintain the general morphology of adherent fibroblast cells. Our results reveal that changes in cortical elasticity are dependent on the duration of exposure. After only 5 min of exposure to CalA there is no change

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**Fig. 4** – Cell height and projected cell areas under various drug treatments. (A) A fibroblast cell transiently expressing PH–PLCδ–EGFP and stained with Hoechst 33342 for the nucleus (scale bar = 20 \( \mu \)m). An orthogonal view along the dotted line (below, scale bar = 6.5 \( \mu \)m). (B) Intensity profile along the dotted line in the orthogonal view was used to determine cell height. (C) Cell height decreases significantly in response to each drug alone, however when used in combination (regardless of the exposure order) cell height increases significantly. (D) Changes in projected cell area did not display any statistically significant trends due to a large degree of variability.
in the cortical elasticity of NIH3T3 cells. However, after 30 min of exposure, increased levels of MLCP reach a point that establishes a significant amount of myosin-II activation and FA assembly. In turn, increased actomyosin tension leads to an accompanying decrease in cell height, giving rise to an increase in apparent cortical elasticity.

On the other hand, depolymerization of MTs releases guanine exchange factor (GEF), which lies upstream of the ROCK/MLC/Myosin-II pathway (Fig. 5B) [53,54]. MTs are dynamic structures that undergo constant polymerization and depolymerization. As nocodazole interferes with MT assembly alone, it is clear that intact MTs must undergo normal depolymerization dynamics before a mechanical effect is observed. We observed that nocodazole treatment led to a rapid increase in FA size and a corresponding decrease in cell height, resulting in a subsequent increase in cortical elasticity, similar to that seen in the case of

Fig. 5 – Possible mechanisms accounting for observed changes in cortical elasticity, FA size and cell height in fibroblasts during contractile activation. (A) The rho-dependent kinase pathway has been well characterized and is involved directly in the regulation of actomyosin contractility and FA assembly. CalA is an inhibitor of myosin light chain phosphatase (MLCPase) causing increased levels of phosphorylated MLC (MLCP), effectively leading to an over-activation of myosin-II. (B) Nocodazole is known to disrupt MTs, releasing guanine exchange factor (GEF), which is upstream of the rho-dependent kinase pathway seen in (A). (C) Cells are assessed at the 0, 5 and 30 min incubation times for the drugs. In the isolated cases, at 0 min, the cell heights ($h$) for both CalA- and nocodazole-treated cells appeared to be comparable to that of the untreated case ($h_0$). After only 5 min of exposure to CalA there is no change in cell height ($h = h_0$). However, cells subjected to 5 min of nocodazole led to the rapid increase in FA size and concomitant loss in cell height ($h < h_0$). Moreover, after 30 min of exposure to CalA, a significant amount of myosin-II activation is established, this induces FA assembly. In turn, the increased actomyosin tension inside the cell leads to an accompanying decrease in cell height ($h < h_0$). After 30 min of exposure to nocodazole, MTs are completely depolymerized which results in an increase in cell height compared to the untreated case ($h > h_0$). When CalA and nocodazole are used sequentially (regardless of the exposure order), there is a large contractile stress that is generated after CalA (30 min) treatment that acts against the internal cellular structures, including the MT network. This leads to an increase in FA size and a corresponding decrease in cell height ($h < h_0$). After 5 min of nocodazole treatment, the MT network is largely depolymerized. We speculate that at this point, a large amount of CalA induced contractile stress may be transmitted largely to FA sites. We hypothesize that tension above a given threshold may result in FA disassembly which leads to the observed decrease in FA size and corresponding increase in cell height $h > h_0$. 

Myosin-II pathway (Fig. 5B) [53,54], MTs are dynamic structures that undergo constant polymerization and depolymerization. As nocodazole interferes with MT assembly alone, it is clear that intact MTs must undergo normal depolymerization dynamics before a mechanical effect is observed. We observed that nocodazole treatment led to a rapid increase in FA size and a corresponding decrease in cell height, resulting in a subsequent increase in cortical elasticity, similar to that seen in the case of
CalA treatment. However, this phenomenon took place within 5 min. After 30 min of exposure, MTs were completely depolymerized, resulting in an increased cell height and corresponding decrease in apparent cortical elasticity, consistent with previous studies [55]. In these individual cases, the known biochemical signaling pathways that are affected result in structural and morphological remodeling of the cell, and corresponding changes in cortical elasticity (Fig. 5C).

Contractile activation, promoted by CalA treatment, resulted in an increase in cortical elasticity. However, this phenomenon was significantly diminished when MTs were depolymerized either before, or after, treatment. Although both CalA and nocodazole can lead to an increase in cortical elasticity when used in isolation, the sequential exposure of both drugs does not lead to an additive increase in cortical elasticity. In order to explain this counter-intuitive result, we speculate that after CalA treatment, a large contractile stress is generated, which acts against the internal cellular structures, including the MT network. This tension leads to the recruitment of FA proteins, leading to an increase in FA size. However, upon the rapid loss of MTs a large portion of the contractile stress is then transmitted to the FAs. It is feasible that FAs may become destabilized and decrease in size when exposed to a contractile stress above a given threshold. We reason that in this case, FA sites lower than a critical size will no longer provide enough resistance against an increase in contractility [56]. FAs are well known to shrink and grow in response to tension [57,58]. Our results suggest that there may be an upper bound to tension-induced FA growth, beyond which FAs will break down. The exact biochemical mechanism for this is unclear at present and will require much more experimentation in order to determine fully. However, in this study FA sites were observed to be much smaller after sequential treatment with both drugs (regardless of order), occurring concomitantly with an increase in cell height and a decrease in cortical elasticity. As mentioned above, changes in cell height are reflected in the apparent cortical elasticity. We hypothesize that the absence of an intact MT network may result in the transmission of a large amount of contractile stress to FA sites, stimulating their disassembly. Biochemical signaling leads to structural changes in the cell, which influences its effective mechanical properties when measured with AFM. Therefore, both biochemical and physical mechanisms appear to be governing the changes in cortical elasticity.

Conclusions

Increasingly evident is the close relationship between mechanotransduction and mechanosensitive signaling pathways [29–31]. Our results reveal that the mechanical properties of the cell cortex are governed by a complex interplay between cytoskeletal and FA remodeling that is highly time dependent. These results show that the stimulation of myosin-II mediated contractility, via myosin-II over-activation or MT depolymerization, leads to increased cortical elasticity. Surprisingly, the sequential stimulation of both pathways did not lead to an additive response, possibly due to FA site sensitivity to the magnitude of contractile stress. Of course, cell type, drug concentration, and the mechanical and biochemical properties of the substrate all play a role in the response [5,12–14,17,18,34,41,59]. Therefore, one must take care when considering the mechanical responses of cells to particular pharmacological agents. Under the conditions in this study, we have demonstrated a dynamic interplay between cytoskeletal remodeling and actomyosin contractility. These processes modulate FA area and cell height, leading to dynamic, time-dependent changes in cortical elasticity of fibroblast cells.

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