Drug-Induced Changes of Cytoskeletal Structure and Mechanics in Fibroblasts: An Atomic Force Microscopy Study

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ABSTRACT The effect of various drugs affecting the integrity of different components of the cytoskeleton on the elasticity of two fibroblast cell lines was investigated by elasticity measurements with an atomic force microscope (AFM). Disaggregation of actin filaments always resulted in a distinct decrease in the cell's average elastic modulus indicating the crucial importance of the actin network for the mechanical stability of living cells. Disruption or chemical stabilization of microtubules did not affect cell elasticity. For the f-actin-disrupting drugs different mechanisms of drug action were observed. Cytochalasins B and D and Latrunculin A disassembled stress fibers. For Cytochalasin D this was accompanied by an aggregation of actin within the cytosol. Jasplakinolide disaggregated actin filaments but did not disassemble stress fibers. Fibrous structures found in AFM images and elasticity maps of fibroblasts could be identified as stress fibers by correlation of AFM data and fluorescence images.

INTRODUCTION

The atomic force microscope (AFM), invented in 1986 (Binnig et al., 1986), has gained some importance for studying biological systems. It is unique because it has proven to be a suitable instrument for imaging biological samples at sub-nanometer resolution in their natural aqueous environment (Drake et al., 1989; Müller et al., 1995; Radmacher et al., 1992). The great potential of the AFM for investigations of living cells was soon recognized. In a pioneering experiment, Henderson et al. (1992) observed the dynamic behavior of the cytoskeleton of living glial cells in AFM contact mode. Qualitative measurements of mechanical properties of living cells were introduced in 1994 by Hoh and Schoenenberger (1994). Dynamic processes such as the activation of platelets (Fritz et al., 1994) and the exocytosis could be followed in time course (Schneider et al., 1997). Information on cellular processes can be derived from the mechanical properties of living cells (Bereiter-Hahn and Lüers, 1994; Elson, 1988). In particular, elasticity measurements provide valuable insights into various dynamic cellular processes such as cell migration (Rotsch et al., 1999) and cell division (Dvorak and Nagao, 1998). Tao and Lindsay were the first to utilize the AFM as a tool for quantitative determination of local elastic properties (Tao et al., 1992). Two-dimensional mapping of the sample elasticity is achieved by recording force curves while the tip is raster-scanned across the sample. This mode of operation has been called force mapping (Radmacher et al., 1994). From the force curves one can calculate the cell topography at different loading forces as well as the local elastic or Young's modulus according to the Hertzian model for elastic indentations (Hertz, 1882; Sneddon, 1965). This has already been applied to bone and bone marrow (Tao et al., 1992), gelatin (Domke and Radmacher, 1998; Radmacher et al., 1995), human platelets (Radmacher et al., 1996), chicken cardiocytes (Hofmann et al., 1997), Kupffer cells (Rotsch et al., 1997a), and MDCK cells (A-Hassan et al., 1998; Hoh and Schoenenberger, 1994).

Dynamical rearrangements in the cytoskeleton enable cells to migrate, to divide, or to maintain their shape (Lee et al., 1993; Stossel, 1993). Therefore, in particular the actin network is supposed to play a major role in determining the mechanical properties of living cells. The importance of actin polymerization and depolymerization for such processes can be understood by studying the effects of drugs like cytochalasin, Latrunculin A, or jasplakinolide that bring about changes in the state of actin polymerization (Bubb et al., 1994; Cooper, 1987; Spector et al., 1983). In particular, effects of cytochalasins on cell elasticities have already been investigated, though not quantified and laterally resolved, by using the cell poker (Petersen et al., 1982).

In this study we wanted to prove the crucial importance of the actin network for the mechanical stability of living cells. The influence of several f-actin-disassembling drugs on cell elasticity is compared and discussed in terms of different mechanisms of drug action. For some of the drugs concentration dependencies were tested. Similar experiments were performed with drugs affecting the integrity of the microtubule network. Correlation of elasticity measurements and fluorescence imaging enabled us to identify the structures found in the elasticity maps.

MATERIALS AND METHODS

Tissue culture

3T3 and NRK fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin (Gibco Life Technologies, Eggenstein, Germany). For the experiments the cells were plated in untreated standard 35-mm plastic petri

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dishes (Nunclon, Naperville, IL) 1 to 3 days before the experiment and kept in an incubator at 37°C in a 5% CO_2 atmosphere. AFM measurements were performed without exchanging the solution.

Fluorescence microscopy

Before fluorescent staining cells were fixed for 10 min in 10% glutaraldehyde in PBS solution, rinsed 3× with PBS, permeabilized for 3 min in a 0.2% Triton X-100 in PBS solution and again rinsed 3× with PBS. Then, rhodamin-phalloidin dye was applied at a concentration of 1:200. After typically 1 h of incubation at room temperature, excess dye was removed by rinsing again 3× with PBS. All chemicals were obtained from Sigma (Deisenhofen, Germany).

Fluorescence images were acquired with a cooled CCD camera (Visicam, Visitron, München, Germany) mounted on an inverted optical microscope (Axiovert, Zeiss, Oberkochen, Germany).

Atomic force microscopy

A commercial stand-alone AFM (Bioscope, Digital Instruments, Santa Barbara, CA) was used for imaging and elasticity measurements. We used very soft cantilevers with spring constants of about 8 mN/m (Microlever/Sharp Microlever, Park Scientific, Santa Clara, CA) as determined by the thermal noise method (Butt and Jaschke, 1995). Cantilevers had tip semiangles of 35° and 18° (Figs. 1 *a-d*, 3, and 4), respectively. To allow convenient positioning of the tip above the cells the AFM was put on top of an inverted optical microscope (Axiomat, Zeiss). Petri dishes were mounted in a custom-built clamp that was magnetically attached to the microscope table. The table could be heated from below with resistors. The AFM was covered with a gas-tight PMMA box that could be flooded with CO_2. Thus, we were able to maintain optimum environmental conditions for the cells throughout the experiment (5% CO_2, 37°C). Isolation from the building's vibrations was achieved by suspending the whole equipment from the laboratory's ceiling with bungee cords.

Imaging was done in contact mode exclusively. Elastic or Young's moduli were derived from force curves. In a force curve the deflection of the AFM tip is recorded as a function of its vertical or z position while it approaches the sample. To measure elastic moduli quantitatively and laterally resolved we operated the AFM in the force mapping mode, also called force volume (Radmacher et al., 1994), i.e., two-dimensional arrays of force curves were recorded while raster-scanning the tip across the sample. Force maps were typically recorded with a frequency of 10 Hz at a resolution of 64 × 64 curves. With these parameters it takes approximately 20 min to obtain a single force map.

From the force curves Young's moduli can be calculated according to Sneddon's modification of the Hertzian model of elastic indentations (Johnson, 1994; Sneddon, 1965). Discussions on the applicability of the

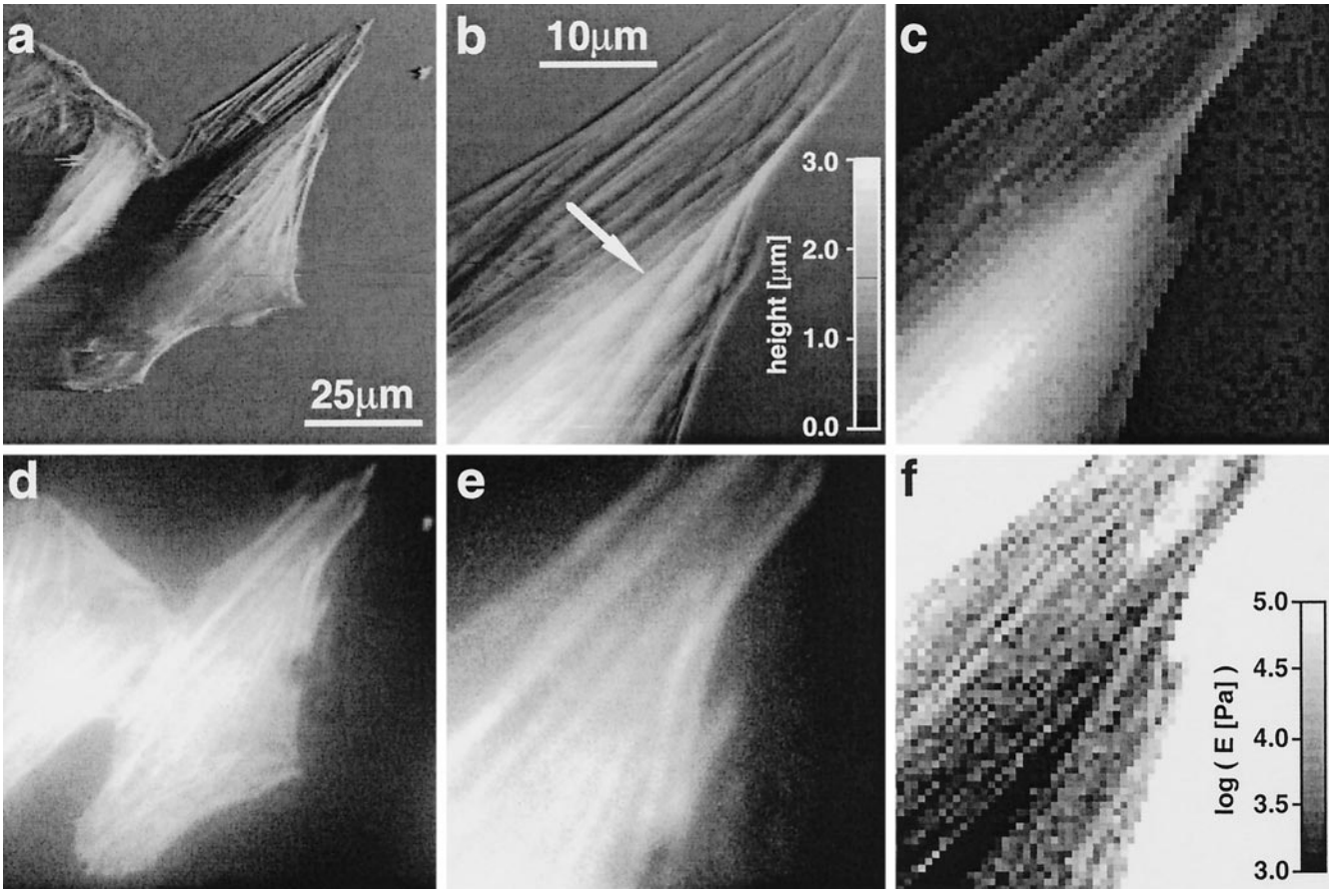


FIGURE 1 Correlation of AFM images, elasticity maps, and fluorescence images to identify fibrous structures as stress fibers. (*a*) AFM deflection image of a living NRK fibroblast. (*b*) Height image at higher magnification, arrow pointing to fibrous structures, presumably stress fibers, (*c*) Height image at zero loading force calculated from a force map of the same region, no fibrous structures present proving these structures to be underneath the membrane. (*d, e*) Fluorescence images of the regions shown in (*a*) and (*b*), actin network labeled with rhodamin-phalloidin after the AFM experiment. (*f*) Elasticity map corresponding to (*c*), fibrous structures possess increased Young's moduli.

model can be found in previous publications (Domke and Radmacher, 1998; Hofmann et al., 1997; Rotsch et al., 1997a, 1999). Here, we restricted data analysis to a very low force range (typically 160–400 pN) to minimize the influence of the underlying stiff substrate. This calculation yields not only values for the local Young's modulus but also the position of the contact point within a force curve. Thus, we can conclude for the real height of the sample. The analysis of the force curves is discussed elsewhere in great detail (Domke and Radmacher, 1998). Here we employ a slightly modified approach as briefly described in a recent publication (Rotsch et al., 1999).

Experimental treatments

To measure the influence of cytoskeletal components on the elasticity of living cells we used drugs to disassemble these structures: Cytochalasins B and D, Phalloidin, Taxol, Colchicine (Sigma), Latrunculin A, and jasplakinolide (gifts from Ilan Spector). All drugs were dissolved in dimethylsulfoxide (DMSO) and diluted in DMEM to yield stock solutions of 10 to 20 times the working concentration. The petri dishes contained 1.8 to 1.9 ml of medium so that by addition of 100 to 200 μl of the stock solutions the desired concentrations could be obtained. However, for this method the drug delivery to the cells is diffusion-limited (Rotsch et al., 1997a). To circumvent this in some cases we either completely exchanged the medium with one that contained the drug at the desired concentration (Fig. 7) or we repeatedly aspired half of the medium into a pipette and back into the petri dish after adding the drug stock solution (Fig. 3) to mix the fluid in the petri dish.

RESULTS

AFM images shown in this publication are deflection images, except for Figs. 2 *b* and 5 *a* (contact height) obtained in AFM contact mode to better visualize small corrugations of the sample surface (Putman et al., 1992).

NRK as well as 3T3 fibroblasts were treated with various drugs acting on different components of the cytoskeleton. Generally, we observed similar effects on both cell types for a given drug at a given concentration.

The results did not depend on the opening angles of the cantilevers used.

Elasticity measurements during drug action were performed by recording time series of force maps on the cells and analyzing the force curve data off-line. In all experiments we recorded a reference force map before adding the drug. In this publication all elasticity maps are encoded in gray shades with logarithmically scaled Young's moduli.

Identification of fibrous structures

An AFM deflection image of a living NRK fibroblast cell is shown in Fig. 1 *a*, the lower half of which appears less structured due to very low applied loading force. At higher magnification we observed cable-like structures in the contact height image, mostly parallel to the longer axis of the cell (Fig. 1 *b*, *arrow*). The cell surface appears distinctly smoother in the height image at zero loading force (Fig. 1 *c*) that was calculated from a force map of the same region (see Materials and Methods). The corresponding elasticity map is shown in Fig. 1 *f*. Here, the fiber-like structures show up as regions of increased elastic modulus. Presumably, the observed structures correspond to stress fibers. To prove this assumption we labeled the actin network of these cells with rhodamin-phalloidin after the AFM experiment and correlated the AFM images and elasticity maps with fluorescence images of the respective regions, as shown in Fig. 1, *d* and *e*.

Generally, NRK cells were found to possess a more pronounced network of these fibrous structures compared to 3T3 cells.

Time series

Control experiments

Several experiments were carried out to prove that the observed effects are caused by the drugs.

During extended periods of AFM imaging no retraction of lamellipodia or disappearance of fibrous structures was observed (five experiments). This is shown in Fig. 2, *a* and *b*, for a 3T3 fibroblast. However, although the total number of fibers did not change distinctly throughout the experiment and lamellipodia did not retract, gradual cytoskeletal rearrangement was observed in time course. These processes were less prominent for NRK fibroblasts, indicating that these cells are less motile.

Continuous force mapping of cells in pure medium (five experiments) yielded no effect on elasticity after 3 h as demonstrated in Fig. 2, *c* and *d*, for a 3T3 fibroblast. In the drug experiments, DMSO is present as a solvent for the agents (compare Materials and Methods). In two control experiments with medium containing only DMSO at the respective concentrations we also found no effect on elasticity. Sample elasticity maps of an NRK fibroblast are shown in Fig. 2, *e* and *f*.

Furthermore, measurements during treatment with phalloidin or drugs affecting the integrity of the microtubule network can be considered control experiments (see below). In these cases, again, no effects on elasticity became apparent. Thus, we conclude that the observed changes in elasticity during treatment with Cytochalasin B and D, Latrunculin A, and jasplakinolide were caused by the influence of the drugs.

Cytochalasin B

Fig. 3 shows the effect of Cytochalasin B in a time series of AFM deflection images of a flat lamellipodium of a 3T3 fibroblast. The scan rate is 1 Hz, leading to an imaging time of 5 min per frame. Cytochalasin B was applied at a concentration of 10 μM and was thoroughly distributed by means of a pipette after the first frame (Fig. 3 *a*) was completed. As expected, a gradual degradation of actin filaments accompanied by a retraction of the whole lamel-

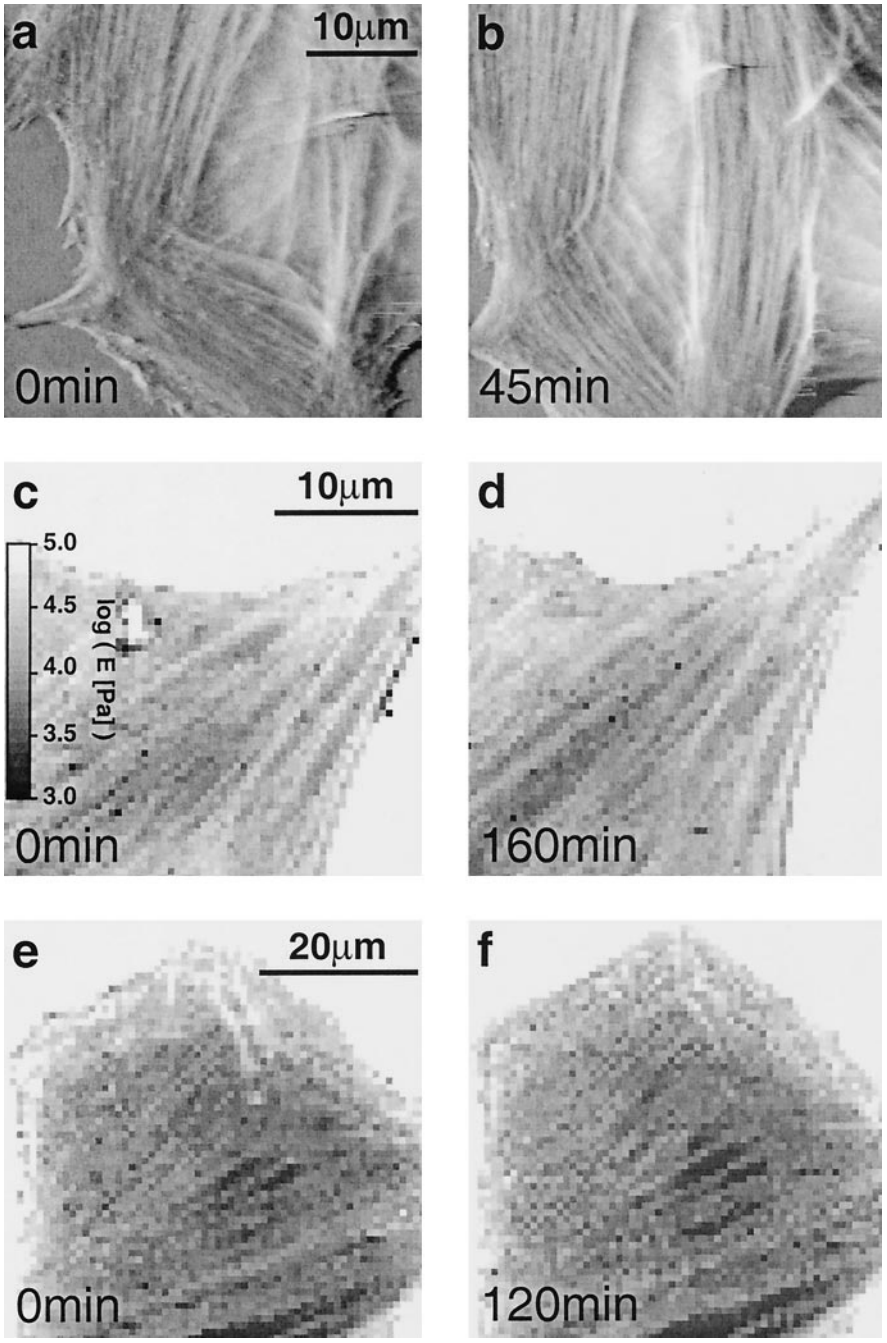


FIGURE 2 Control experiments proving the observed effects to be caused by the drugs. (*a, b*) During continuous AFM imaging of a 3T3 fibroblast the number of fibrous structures increased and lamellipodia were extended in time course. (*c, d*) No effect on elasticity after 3 h of continuous force mapping of a 3T3 fibroblast in pure medium. (*e, f*) No effect on elasticity after 3 h of continuous force mapping of an NRK fibroblast in medium containing DMSO.

lipodium can be observed. Smaller filaments disappear almost completely within the first 10 to 15 min, whereas larger structures, presumably stress fibers, last up to 30 min (see *arrows* in Fig. 3, *a* and *g*). No disappearance of filaments and fibers was observed in control experiments (Fig. 2, *a* and *b*).

The effect of Cytochalasin B on the Young's modulus of living cells is shown in Fig. 4 in a time series of elasticity maps of a 3T3 fibroblast. Cytochalasin B (10 μM) was applied after a reference force map was completed (Fig. 4 *a*). In this figure stress fibers can clearly be seen (*arrow*). Within 60 min the Young's modulus already decreased by a factor of 2.2, indicating that a loss of actin filaments dramatically affects the cell's mechanical stability. Stress fibers are still present. However, after 120 min the stress fibers disappear as well (Fig. 4 *g*). Throughout the experiment the average Young's modulus of the cell decreased by a factor of 3.1.

Values for the reduction in elasticity were obtained by analyzing histograms of areas of interest in the elasticity maps. As an area of interest we picked the largest region around the nucleus that did not contain flat parts like lamel-

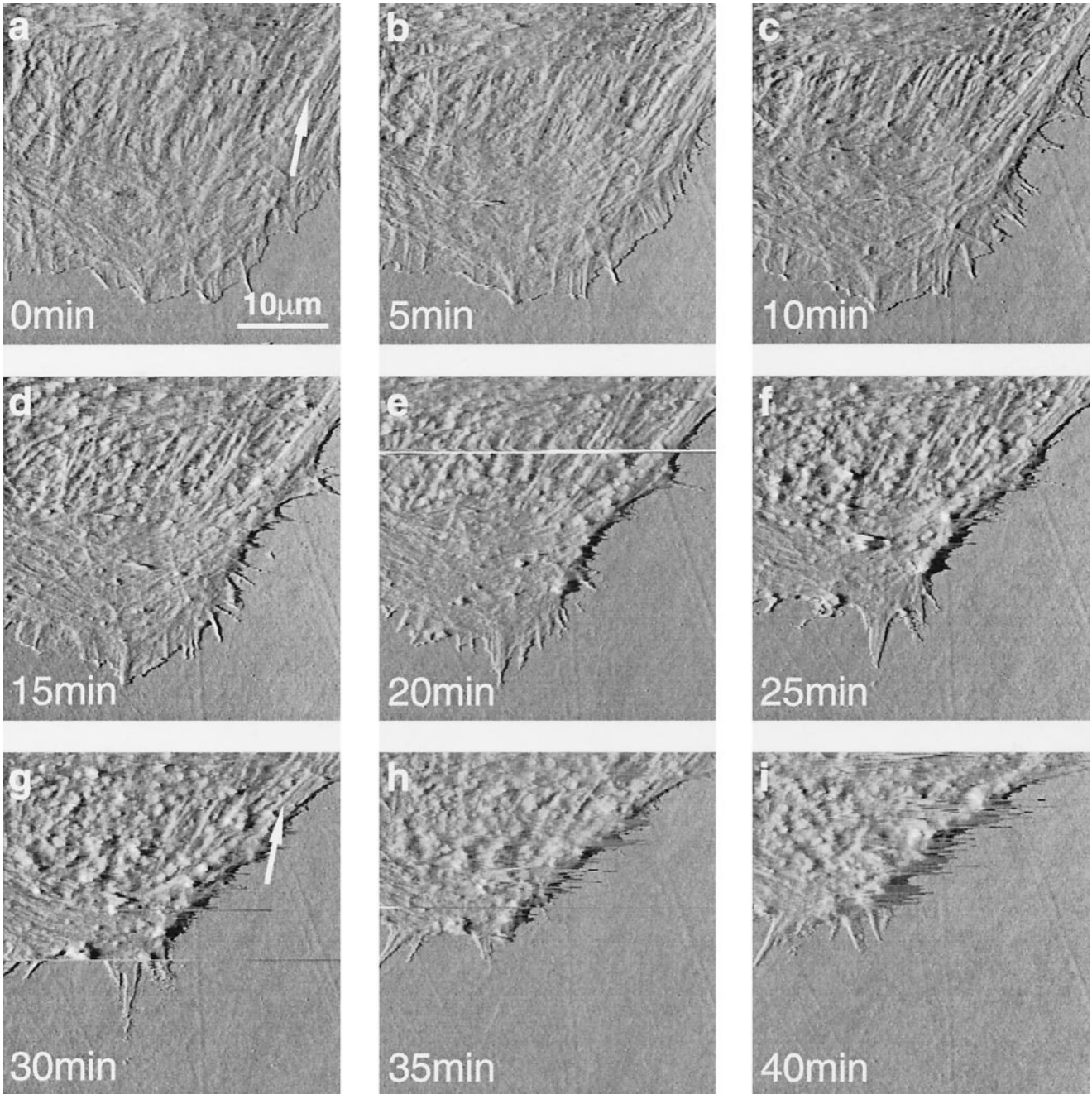


FIGURE 3 Time series of AFM deflection images of an NRK fibroblast showing disaggregation of the actin network in the lamellipodium of a 3T3 fibroblast by 10 μM Cytochalasin B. (*a*) Reference before adding Cytochalasin B. (*b-i*) After Cytochalasin B has been added, actin network disappears gradually as the lamellipodium retracts, stress fibers last longer than smaller structures; see *arrows* in (*a*) and (*g*).

lipodia. The flat parts were excluded from the analysis because there the elastic moduli is often overestimated due to the influence of the underlying substrate. Flat regions were excluded by comparison with the corresponding height images. For different areas of interest in Fig. 4 the elasticity decrease varied between 3.03 and 3.16.

Treatment of NRK (seven experiments) and 3T3 fibroblasts (11 experiments) with 5 to 20 μM of Cytochalasin B yielded values for the elasticity decrease between 2.6 and 3.1. No apparent differences in the drug effect on the two cell types were observed.

The delay from the time of addition of the drug to measurable effects on cell elasticity seems to depend on the drug concentration. At higher concentrations (50–100 μM) Cytochalasin B affects the cell much faster. Typically, cells round up and detach from the substrate within 10 min (i.e.,

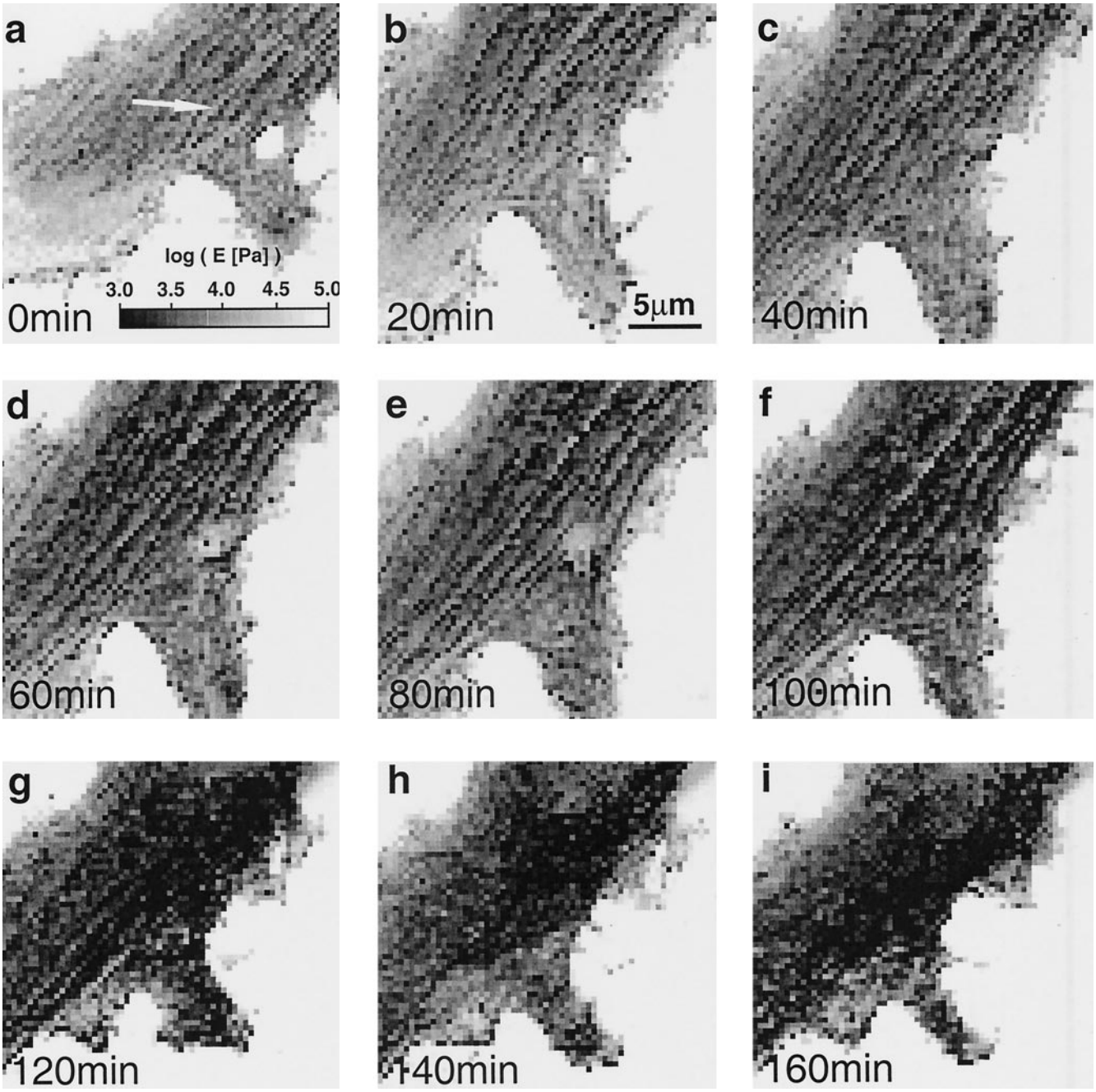


FIGURE 4 Time series of elasticity maps of a 3T3 fibroblast showing disaggregation of the actin network by 10 μM Cytochalasin B. (*a*) Reference; *arrow* points to stress fiber. (*b-i*) Successive elasticity maps show gradual decrease in the cell's Young's modulus; stress fibers last up to 100 min (*f*).

before the first force map is completed). At very low concentrations (1–2 μM) no distinct effects on cell elasticity can be observed within 3 h. However, time constants for the drug action at a given concentration vary over a wide range (20–120 min for 10 μM Cytochalasin B).

Cytochalasin D

A NRK fibroblast that has been treated with Cytochalasin D is presented in Fig. 5. AFM images of the cell recorded before applying the drug are shown in Figs. 5 *a* (height) and 5 *b* (deflection). Stress fibers across the nuclear and perinuclear regions can clearly be seen (*arrows* in Fig. 5 *b*). Again, the fibers do not show up in the height image at zero loading force (Fig. 5 *c*) that was calculated from the reference force map. The corresponding elasticity map is shown in Fig. 5 *d*. After taking this reference force map 10 μM of Cytochalasin D were applied. A slight decrease in elasticity can already be observed in the next data set (Fig. 5 *e*), which was recorded from 0 (bottom) to 20 min (top) after the drug

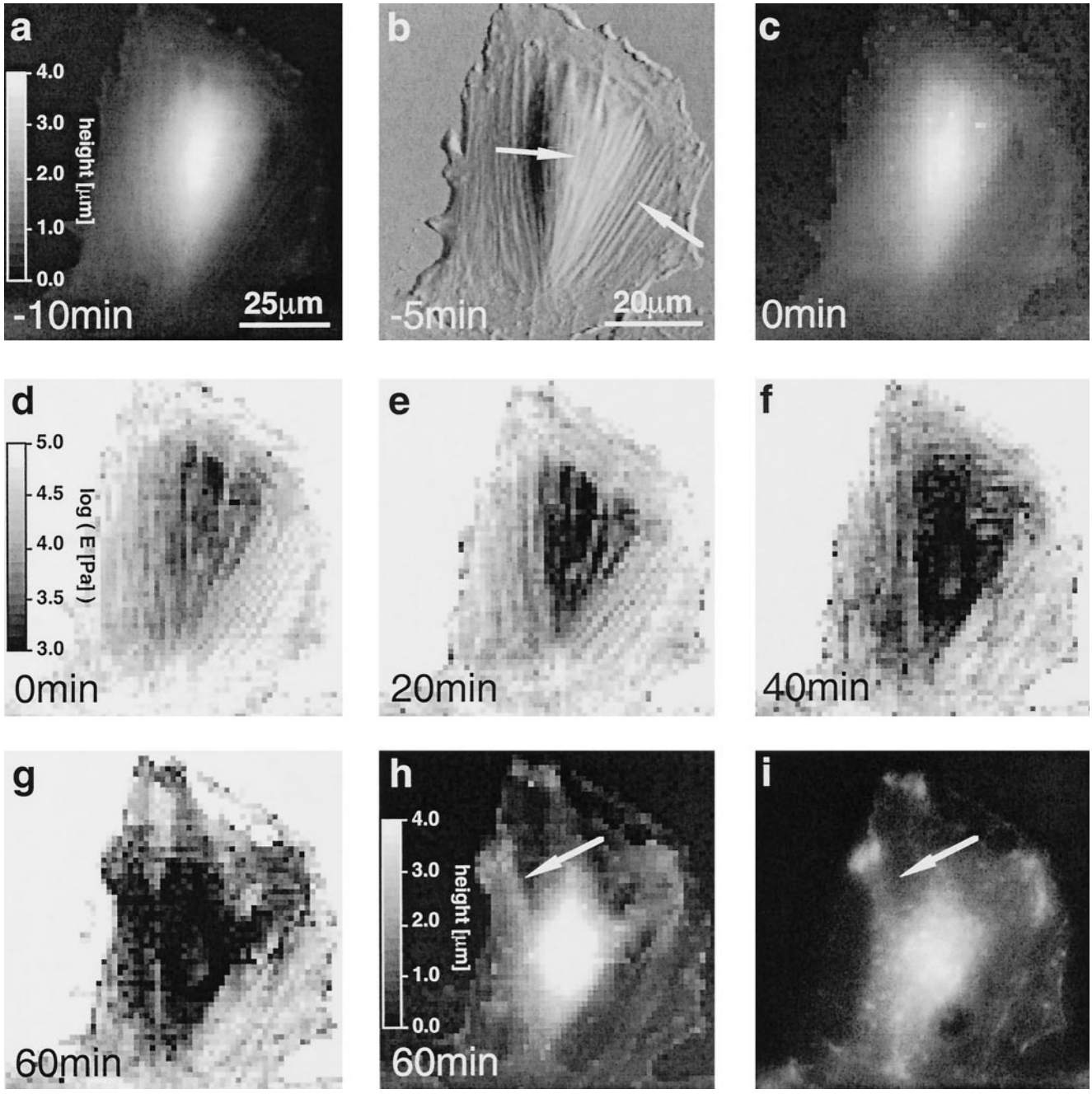


FIGURE 5 Time series of elasticity maps of an NRK fibroblast showing disaggregation of the actin network by 10 μM Cytochalasin D and correlation with AFM and fluorescence images. (*a*) Contact AFM image. (*b*) AFM deflection image; stress fibers are depicted by *arrows*. (*c*) Height image at zero loading force calculated from the reference force map. (*d*) Corresponding reference elasticity map. (*e, f*) Young's modulus decreases gradually within 40 min. (*g*) Elasticity map and (*h*) height image at zero loading force obtained after 60 min showing strong segmentation of the cytosol. (*i*) Corresponding fluorescence images to identify structures in (*g*) and (*h*) as clots of actin. *Arrow* in (*h*) points to a very flat region ($<$500 nm) that correlates with a region devoid of f-actin (*i, arrow*).

was added. During the next 20 min (Fig. 5 *f*) the cell's average Young's modulus decreased by a factor of 2.9 compared to the reference. After 60 min a distinct segmentation of the cytosol could be observed (Fig. 5 *g* (E) and 4 *h* (height at zero loading force)). Immediately after obtaining this data set the cell was fixed, permeabilized, and stained. The resulting fluorescence image is shown in Fig. 5 *i*.

Fifteen experiments were analyzed where 3T3 (five experiments) and NRK (10 experiments) fibroblasts were treated with Cytochalasin D at concentrations between 5

and 30 μM. Values for the elasticity decrease were between 2.6- and 3.2-fold for 3T3 cells and between 2.8- and 3.2-fold for NRK cells.

Concentration dependencies for effects of Cytochalasin D were comparable to what we observed for Cytochalasin B. Cell elasticities were not altered significantly under the influence of 1 μM Cytochalasin D for up to 3 h, whereas cells detached rapidly from the substrate at higher concentrations (50–100 μM).

Latrunculin A

Fig. 6 shows an NRK fibroblast treated with 0.1 μM Latrunculin A. Again, Fig. 6 *a* is a reference force map recorded before adding the drug. No distinct effect on elasticity was measured within the first 40 min (Fig. 6, *b* and *c*). Then, the cell's Young's moduli gradually decreased 2.4-fold (Fig. 6, *d-f*). Stress fibers (*arrow* in Fig. 6 *a*) are disassembled simultaneously. Cell heights remained almost unchanged throughout the experiment.

In three experiments on NRK fibroblasts treated with 0.1 μM Latrunculin A the elasticity decreased between 2.4- and 2.7-fold. A single experiment on a 3T3 fibroblast yielded a reduction of the average elasticity by a factor of 2.7. Concentration dependencies were not tested

Jasplakinolide

A time series of elasticity maps of a 3T3 fibroblast treated with jasplakinolide is presented in Fig. 7. Contact AFM images of the investigated cell were obtained before (Fig. 7, *a* and *b*) and after (Fig. 7 *c*) the elasticity measurements. Note the cell's extended lamellipodia before the drug experiment (*arrowheads* in Fig. 7 *b*) in contrast to the mainly concave cell edges afterward (*arrowheads* in Fig. 7 *c*). The whole medium was replaced by 0.1 μM jasplakinolide in medium after recording the reference force map (Fig. 7 *d*) to possibly overcome the diffusion-limited delay between addition of jasplakinolide and its effect. Nevertheless, no onset of the drug effect on the measured cell elasticity can be observed within 40 min after drug application (Fig. 7, *e* and *f*). Whether or not a slight increase of the elastic modulus in the nuclear region (compare Figs. 7 *d* and 7 *f*) is induced by the drug or is caused by unforced rearrangement of the cell's cytoskeleton remains speculative. After 60 min active regions of the cell start to retract and the cell's average

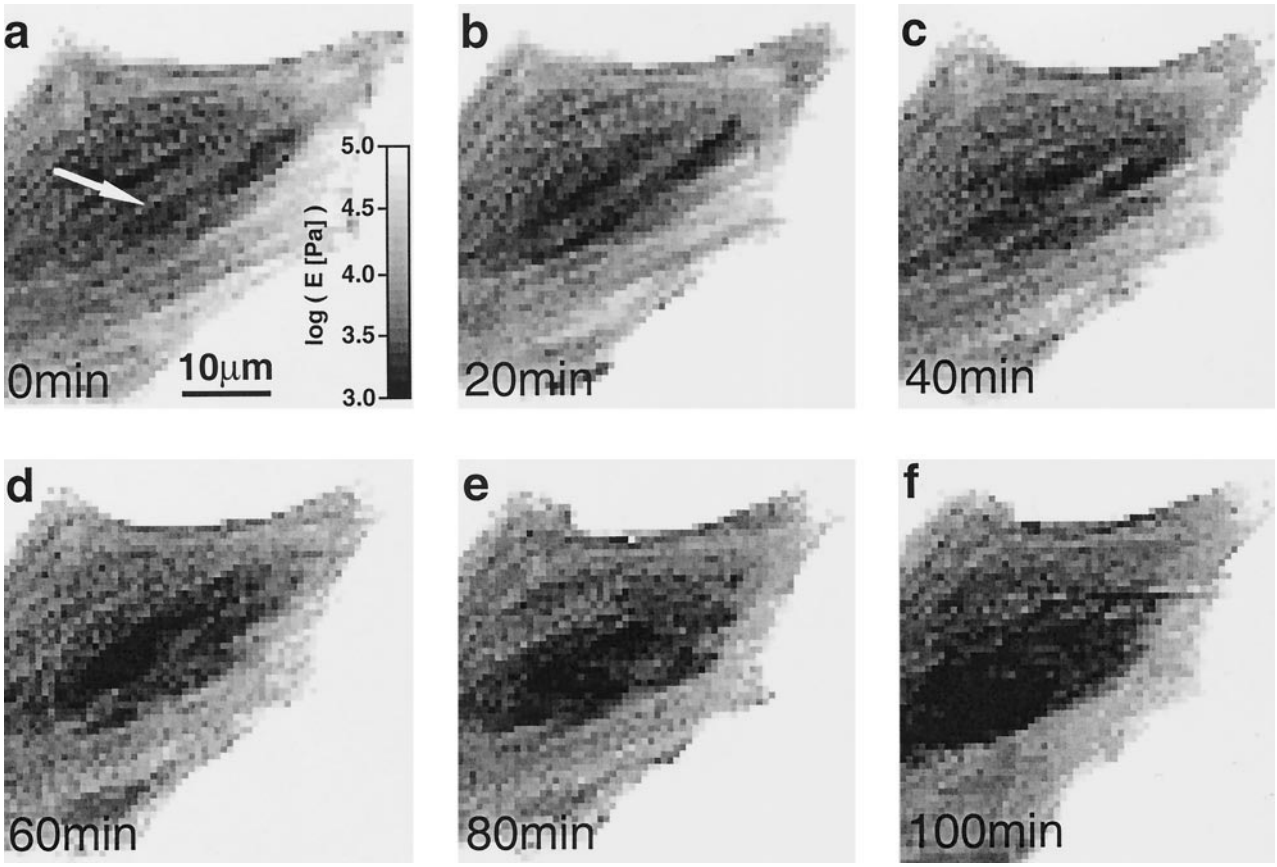
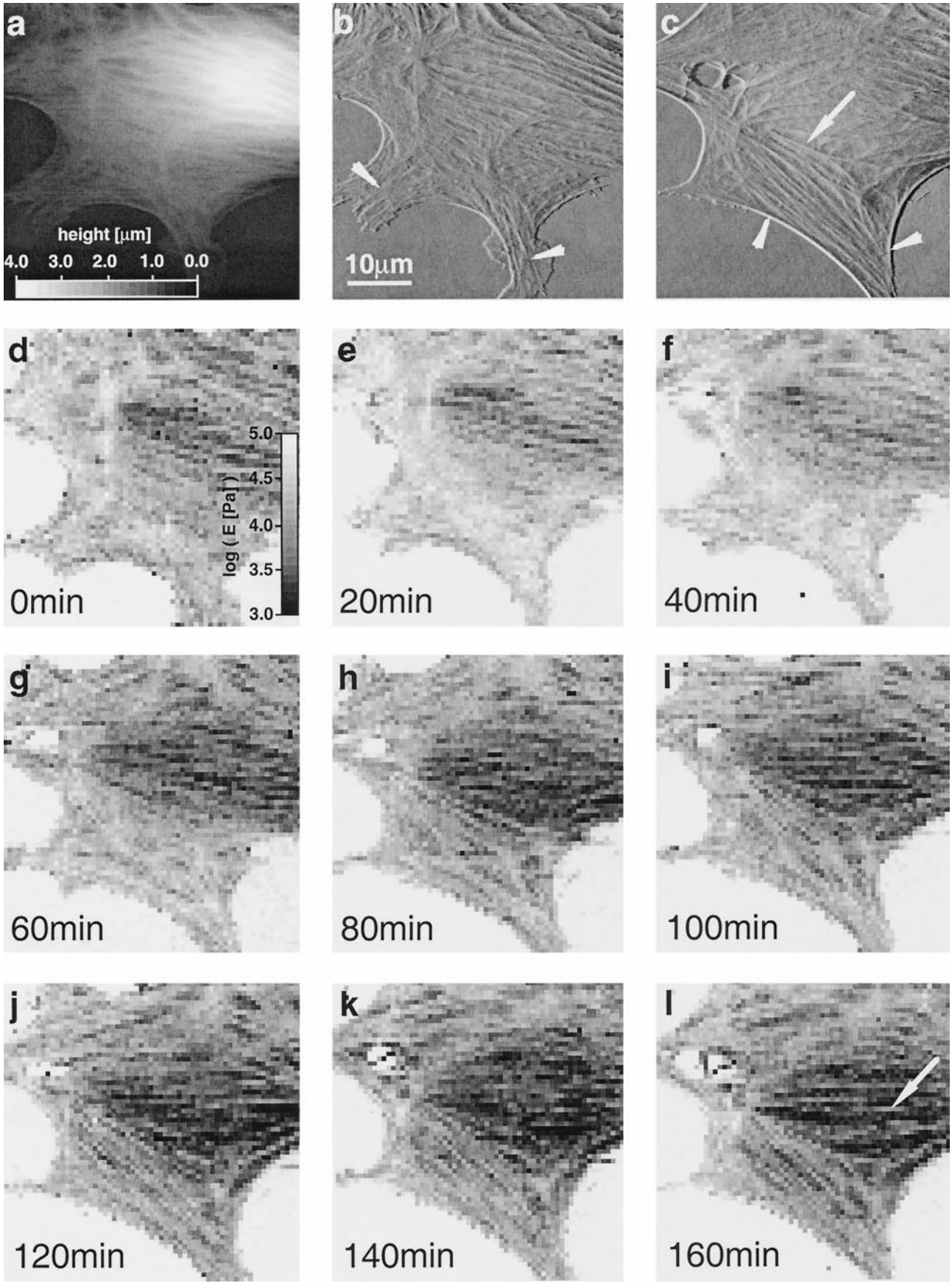


FIGURE 6 Time series of elasticity maps of an NRK fibroblast showing disaggregation of the actin network by 0.1 μM Latrunculin A. (*a*) Reference; *arrow* depicts stress fiber. (*b, c*) No distinct effect within 40 min. (*d-f*) Gradual decrease in the cell's Young's modulus.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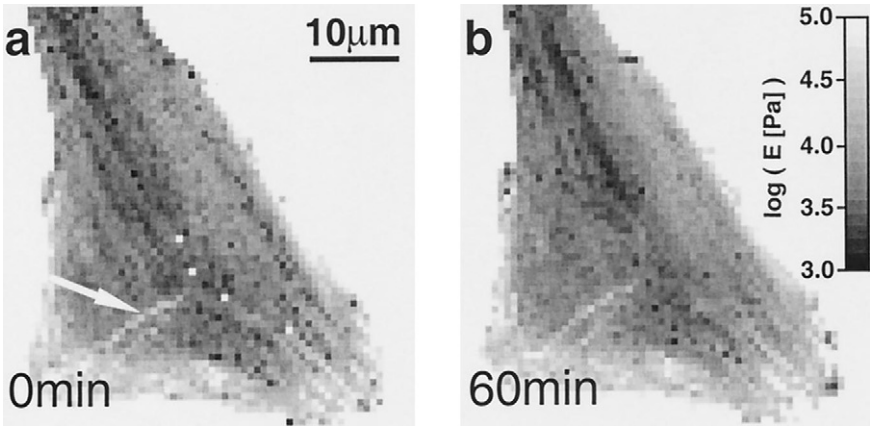

FIGURE 8 Time series of elasticity maps of an NRK fibroblast showing disaggregation of the microtubule network by 100 μM colchicine. (*a*) Reference. (*b*) No effect on cell's Young's modulus after 60 min.

Young's modulus gradually decreases with time (Fig. 7, *g-l*) 2.4-fold (Fig. 7 *l*). However, in contrast to the cytochalasins and Latrunculin stress fibers are not disassembled and can still be observed even after 3 h of drug treatment (*arrows* in Fig. 7, *c* and *l*). It should be noted, though, that the regional distribution of stress fibers does change in time course. This might again reflect metabolic processes that, for instance, accompany cell migration.

Treatment of NRK (six experiments) or 3T3 fibroblasts (three experiments) with 0.05 to 0.5 μM jasplakinolide caused reductions of the cell's average elastic modulus between 2.4- and 2.8-fold for NRK cells and between 2.7- and 2.8-fold for 3T3 cells.

At lower concentrations (0.01 μM), again, no distinct effect on cell elasticity became apparent. However, cells do not detach from the substrate at concentrations up to 10 μM, whereas the average Young's modulus drops almost instantaneously after drug application by a factor of about 6 (value not reliable because the nucleus is pushed through the cytoplasm by the scanning AFM tip, thereby causing distortion of force curves). Stress fibers were still present after 20 min.

Colchicine, colcemide, taxol, and phalloidin

An exemplary dataset for an NRK fibroblast treated with 100 μM colchicine is shown in Fig. 8. After 60 min (Fig. 8 *b*) we did not observe any influence of the drug treatment on cell elasticity compared to the reference elasticity map (Fig. 8 *a*). The cell maintained its shape and stress fibers (*arrow* in Fig. 7 *a*) were not disassembled. Similarly, no effects were measured at concentrations up to 500 μM for colchicine (three experiments), colcemide (two experiments), and phalloidin (two experiments) and up to 100 μM for taxol (three experiments) within 2 h of drug influence, even when the medium was replaced by the drug dissolved in medium.

DISCUSSION

Fibrous structures in AFM images correspond to stress fibers

In AFM images of tightly adherent cells such as fibroblasts, chicken cardiocytes, or glial cells, large fibrous structures across the cell are prominent (Henderson et al., 1992; Hofmann et al., 1997), whereas such structures cannot be found in more motile cells like fish keratocytes (data not shown) or rat liver macrophages (Rotsch et al., 1997a). For glial cells it was shown that these structures are stress fibers (Henderson et al., 1992).

Stress fibers are temporary contractile bundles of actin filaments and myosin that insert at one end into the plasma membrane, forming focal adhesion points. They are responsible for cell contractions and affect the locomotion of the cell (Alberts et al., 1997).

Fibrous structures similar to those in the AFM images can be found in elasticity maps of living cells. In previous publications they were assumed to be stress fibers (Hofmann et al., 1997; Rotsch et al., 1997b, 1999), but this has not yet been proven unequivocally. Therefore, a major goal of this publication was to identify the structures in the elasticity maps.

Due to the softness of living cells the AFM tip will indent the cell, thereby pressing the cell membrane into the cytosol unless the membrane is supported by stiffer structures, causing these structures to appear elevated and therefore to show up in the deflection images (Rotsch et al., 1997b). Thus, a comparison of the contact mode height image (Fig.

FIGURE 7 Time series of elasticity maps of a 3T3 fibroblast showing disaggregation of the actin network by 0.1 μM jasplakinolide. AFM deflection images obtained before (*a* (height) and *b* (deflection)) and after (*c*) the experiment, active lamellipodia turned into stable edges (*arrowheads*), *arrow* in (c) depicts stress fiber. (*d*) Reference elasticity map. (*e, f*) No effect within 40 min. (*g*) Active regions start to retract. (*h-l*) Gradual decrease in the cell's Young's modulus, further retraction of active regions. After 3 h stress fibers are still present.

1 *b*) with the calculated height image at zero loading force from the force map which visualizes the undisturbed cell surface (Fig. 1 *c*) already reveals that the fibers are underneath the membrane. Accordingly, the Young's modulus on top of the fibers is larger, as can be seen by comparing Figs. 1 *b* and 1 *f*.

In Fig. 1, *d* and *e*, actin was fluorescently labeled using rhodamin-phalloidin. Phalloidin binds specifically to filamentous actin, thereby stabilizing the actin filament chemically by inhibiting dissociation of monomers (Coluccio and Tilney, 1984; Frimmer, 1977). This specificity is employed for fluorescent staining: phalloidin mediates the bond between the fluorescent dye rhodamin and filamentous actin. Labeling of short actin oligomers may account for the background in the fluorescence images. The good correlation between the AFM image data (Fig. 1, *a* and *b*), the elasticity map (Fig. 1 *c*) and the fluorescence images (Fig. 1, *d* and *e*) proves that the fibrous structures are stress fibers.

The influence of such fibrous structures now identified as stress fibers on cell elasticity has already been discussed in great detail in previous work (Hofmann et al., 1997).

Note the dependency of the local Young's modulus on cell thickness (Fig. 1, *c* and *f*). This correlation is only observed in very flat regions of the cell and represents an artifact caused by the influence of the underlying substrate. A more detailed discussion of the problem can be found in earlier publications of our group (Domke and Radmacher, 1998; Rotsch et al., 1999).

The comparatively more pronounced network of stress fibers in NRK cells, furthermore, supports the observation that 3T3 fibroblasts are more motile (compare Rotsch et al., 1999).

Long-term experiments are possible

The drug experiments introduced in this publication comprise long-time measurements on living cells with an AFM. Our setup allows the maintenance of optimum environmental conditions for the cells (37°C and 5% CO_2). The restriction to blunt AFM tips (opening semi-angles 18° or 35°) and very low loading forces (<1 nN) ensures that the tip does not penetrate the cell membrane, as demonstrated by Haydon et al. (1996). Thus, we were able to keep the cells viable for at least 3 h despite continuous indentation by the AFM tip and the application of cytotoxic drugs. Even AFM contact mode imaging where additional lateral forces are exerted onto the cell was possible over extended periods of time (Fig. 3). Cell viability has been confirmed in earlier publications by the Trypan blue exclusion test (Rotsch et al., 1997a), but can also be concluded from the continuation of metabolic processes such as cell migration (Rotsch et al., 1999) or lasting adhesion to the substrate.

The duration of the experiments is limited by evaporation of the medium, which results in an increased concentration of salts and proteins in the petri dish. At room temperature, similar drug experiments were performed that lasted more than 6 h. The observed effects were comparable. However, here we present only data from experiments under physiological conditions, in particular at 37°C.

The actin network imparts mechanical strength to living cells

In the drug experiments we did not measure any significant differences in the effects of the actin-disassembling drugs on the elasticity of NRK as compared to 3T3 fibroblasts. Differences between both cell lines concern the general morphology, in particular the generally better developed network of stress fibers in NRK fibroblasts, making them less motile but more stable and better adherent to the substrate and therefore easier to handle in AFM experiments. (That is, for NRK cells less care need be taken to keep the applied loading force low in order to avoid detachment of the cells from the substrate.) The results obtained on the effects of drug treatment are also in agreement with earlier measurements on chicken cardiocytes (Hofmann et al., 1997) and Kupffer cells (Rotsch et al., 1997a), which indicates the observed effects to be of a general nature. The comparably large decrease in the average Young's modulus of Kupffer cells as a consequence of Cytochalasin B treatment could have been caused by floating of microvilli or lamellipodia, respectively. Otherwise we always found the disaggregation of the actin network in various cell types to result in a reduction of the cell's average Young's modulus by a factor of approximately 3. This demonstrates the general importance of the actin network for the mechanical properties of living animal cells.

Cytochalasin B and D treatment caused 2.9-fold reductions in the cell's average Young's modulus (average values of 18 and 15 experiments, respectively), Latrunculin A a 2.6-fold decrease (four experiments), and jasplakinolide a 2.5-fold decrease (11 experiments). For a given drug at a given concentration the amount of elasticity decrease varied distinctly between individual experiments (compare Table 1). Thus, it remains a matter for speculation whether these small differences between the effects of the cytochalasins and Latrunculin A and jasplakinolide are significant.

Different mechanisms of drug action were observed

Our measurements of the influence of a chemical disassembly of the actin network on the cell's elastic properties not only allow us to conclude for the general importance of the actin cytoskeleton for the mechanical stability of the cell. Moreover, it is possible to monitor different mechanisms of drug action.

TABLE 1 Effects of drugs

Drug	Known effect*	Drug concentration	Number of experiments	Decrease in elasticity	Delay of drug effect (min)	Effect on stress fibers
Cytochalasin B	disrupts	<1 μM	3	none	–	none
	f-actin	5–20 μM	18	2.6 . . . 3.1-fold	20–120	disrupted
		>50 μM	2	n.a.†	$\ll 20$	disrupted
Cytochalasin D	disrupts	<2 μM	3	none	–	none
	f-actin	5–30 μM	15	2.6 . . . 3.2-fold	20–100	disrupted
		>50 μM	2	n.a.†	$\ll 20$	disrupted
Latrunculin A	disrupts f-actin	0.1 μM	4	1.4 . . . 2.7-fold	40–80	disrupted
jasplakinolide	cell/	<0.01 μM	2	none	–	none
	concentration	0.05–0.5 μM	9	2.4 . . . 2.8-fold	40–120	none
	dependent	1–10 μM	3	(6-fold)	$\ll 20$	none
colchicine, colcemide	disrupts microtubules	≤ 500 μM	5	none	–	none
taxol	stabilizes microtubules	≤ 100 μM	3	none	–	none

*References given in text.
†Cells detached from substrate during experiment.

Cytochalasin D but not Cytochalasin B caused actin aggregation

Cytochalasins are a family of metabolites excreted by various molds. Their principal action is to bind to the fast growing plus ends of actin filaments, thus preventing the addition of actin monomers at this end (Bray, 1979; Cooper, 1987; Sampath and Pollard, 1991). This leads to a depolymerization of the filaments due to the net loss of actin monomers at their minus ends. In many migrating cells the application of Cytochalasin results in a rapid retraction of the leading edge. However, the specificity of cytochalasins to actin is not evident. Cytochalasin B but not Cytochalasin D was found to additionally inhibit monosaccharide transport. Both agents were observed to cause an aggregation of filamentous actin into dispersed felt-like masses enveloping large cystic vacuoles (Brett and Godman, 1984; Godman et al., 1980a,b). Generally, side effects of the cytochalasins apart from actin depolymerization are difficult to exclude.

In Fig. 5 *i* the cell's f-actin, which after Cytochalasin D treatment mostly consists of short, non-cross-linked actin oligomers, was fluorescently labeled with rhodamin-phalloidin. Our measurements reveal a strong correlation between f-actin-rich regions, areas which are soft (Fig. 5 *g*) compared to the reference force map and high parts of the cell (Fig. 5 *h*). This proves the observed segmentation of the actin cytoskeleton.

Regions of the cell devoid of f-actin according to the fluorescence data are typically <500 nm high (*arrows* in Fig. 5, *h* and *i*). In regions of untreated cells with thicknesses of about 500 nm we obtain reasonable values for the elastic modulus because at the low forces used for analyzing the force curves the indentation of the AFM tip into the cell is sufficiently small (<230 nm for $E = 5$ kPa) to rule out the influence of the underlying substrate (Domke and Radmacher, 1998). However, if f-actin is disassembled the cell's resistance against mechanical load is reduced and the indentation will be larger for a given loading force. Thus, the elastic moduli measured on such flat parts of the cell will be larger than the true values according to the influence of the substrate, even if force curves are analyzed in the lowest possible force range (see Materials and Methods). This effect will be more pronounced the softer the sample is. Therefore, the flat regions appear stiffer after drug treatment, although the true elastic modulus of the cell was reduced.

NRK as well as 3T3 cells treated with Cytochalasin D at concentrations of 530 μM always showed a strong segmentation in the cytosol (Fig. 5, *g* and *h*). The observed felt-like masses could be confirmed to consist of filamentous actin by correlation with fluorescence data (Fig. 5 *i*). Typically, segmentation was preceded by a softening of the cell indicating the disruption of actin filaments (Fig. 5 *f*) and occurred simultaneously with the disappearance of stress fibers. Thus, we can conclude that the aggregated actin filaments originate from stress fibers.

At the same concentrations the decrease in the average Young's modulus caused by Cytochalasin B and D, respectively, was comparable (Figs. 4 *f* and 5 *f*) for both agents within the given range of concentration. This suggests that both are similarly potent inducers of f-actin disruption. However, we never observed such an aggregation of actin as a consequence of Cytochalasin B treatment which indicates Cytochalasin D to more severely disturb other cellular processes.

Petersen et al. (1982) used the cell poker to measure the elasticity decrease merely in the perinuclear region of the cell as a consequence of Cytochalasin B treatment. They suspected nuclear elasticities were mainly determined not by the actin network but by nuclear components or other structures prominent in the perinuclear region. In contrast to

these observations we measured similar effects of the drug on elasticity across the whole cell body. This is not contradictory and can be explained by our restriction to very low cantilever deflection values in data analysis. The AFM tip indents a cell with a Young's modulus of 10 kPa by <260 nm. Thus, the measured elastic moduli may to a large degree be determined by the very thin actin cortex in addition to the contribution of the underlying nucleus.

Cytochalasin B and Latrunculin A effects are similar

Latrunculin A, another potent inhibitor of actin polymerization, can be isolated from the Red Sea sponge *Latrunculia magnifica* (Spector et al., 1983). It forms 1:1 complexes with actin monomers and thereby shifts the equilibrium between the monomers and f-actin (Coue et al., 1987; Spector et al., 1986). This leads to a disruption of the actin cytoskeleton in various cells (Lyubimova et al., 1997; Oliveira et al., 1997; Spector et al., 1989).

Latrunculin A affects the elasticity of fibroblasts phenomenologically similar to Cytochalasin B, though at distinctly lower concentrations of 0.1 μM. Again, an overall decrease in the average Young's modulus by a factor 2.6 was observed (Fig. 6, *a* and *f*). Bundling of actin filaments is likely to reduce the treadmilling of the filaments and thus Latrunculin A should disrupt stress fibers much slower than the surrounding network of single actin filaments. This hypothesis is confirmed by our measurements. In Fig. 6 *d* we already observe a distinct decrease in elasticity, although stress fibers are still present.

In contrast to our measurements on rat liver macrophages (Rotsch et al., 1997a) which are devoid of stress fibers the effect of Latrunculin A did not occur suddenly. The more gradual elasticity decrease as observed for the fibroblasts (Fig. 6) may be caused by the slower disruption of stress fibers. The restriction of the Latrunculin A effect on the macrophages to the perinuclear region (Rotsch et al., 1997a) can again be explained by distinctly larger indentations of the AFM tip in these experiments (up to 900 nm, caused by sharper AFM tips, larger deflections, and softer cells).

Jasplakinolide does not disassemble stress fibers

Jasplakinolide, which is also derived from marine sponges (*Jaspis johnstoni*) is a recently discovered agent that affects the actin cytoskeleton. Information on the effects of the drug are contradictory and indicate concentration and cell type dependencies. It disrupts the actin cytoskeleton of human prostate carcinoma cells at concentrations of about 0.1 μM (Duncan et al., 1996; Senderowicz et al., 1995). The antiproliferative activity against these cells make it a potential antitumor agent. In vitro jasplakinolide induces actin polymerization (Bubb et al., 1994). An increased rigidity of neutrophils caused by treatment with 10 μM jasplakinolide was indicated by resistance of the cells to pipette aspiration (Sheikh et al., 1997). In liver endothelial cells concentrations of jasplakinolide as low as 10 nM lead to a disruption of actin network and stress fibers and to an aggregation of f-actin (Braet et al., 1998).

In our experiments Cytochalasin B and D and Latrunculin A treatment initially lead to a general softening of the cell in regions devoid of stress fibers. Although the integrity of the network of cross-linked f-actin is already severed, bundles of actin filaments, i.e., stress fibers, appear to not yet be affected by the drugs (Figs. 3 *c*, 4 *d*, 5 *e*, and 6 *d*). A lower time constant for the turnover of actin monomers in bundled filaments might explain the delayed effect of the cytochalasins and Latrunculin A on stress fibers. The cytochalasins and Latrunculin A shift the equilibrium between actin filaments and monomers by capping the plus ends of the filaments or binding actin monomers, respectively (Coue et al., 1987; Sampath and Pollard, 1991). However, in all respective experiments these drugs were found to be capable of disassembling stress fibers as well as cross-linked f-actin within less than 3 h as confirmed by the total disappearance of stress fibers (Figs. 3 *g*, 4 *g*, 5 *g*, and 6 *e*).

In contrast to these agents jasplakinolide appears not to disrupt stress fibers (Fig. 7, *b* and *c*). Numerous fibers are still present 3 h after addition of jasplakinolide. Cytoskeletal rearrangements during the experiment may originate from metabolic processes.

The different effects of the drugs on stress fibers suggest a fundamental difference in the mechanisms of drug action. Possible mechanisms in agreement with the insensitivity of stress fibers to jasplakinolide are, for instance, a severing of cross-links between actin filaments or a breaking of single actin filaments. However, in other cells, such as liver endothelial cells, jasplakinolide was found to disrupt stress fibers (Braet et al., 1998).

The incapability of jasplakinolide to disrupt stress fibers in fibroblasts may also account for the lasting attachment to the substrate of cells treated with high concentrations of the drug in contrast to the rapid detachment under influence of higher concentrations of the cytochalasins.

Phalloidin could not enter the cells

Phalloidin by itself is a relatively large molecule (789 g/mol) and thus not able to enter the cell through the intact membrane (Coluccio and Tilney, 1984; Frimmer, 1977). For fluorescent staining the membrane therefore has to be permeabilized by a detergent before the application of the dye. Permeabilization of the membrane is likely to influence cell elasticity. In our measurements we did not permeabilize the membrane so that the result can merely be considered a nice control experiment: phalloidin could not get into the cell and no changes in elasticity were observed.

Time constants for the drug effects could not reliably be retrieved

Concentration dependencies for the effects of the cytochalasins and jasplakinolide on cell elasticity were extensively tested. Generally, we observed a faster drug action at higher concentrations but only minor differences in the amount of decrease of the average Young's modulus. The latter holds true only as long as the cell remains fully attached to the substrate. Otherwise floating lamellipodia impede the measurement, resulting in distorted force curves. There seem to be concentration thresholds of 1 to 2 μM for Cytochalasin B, 1 μM for Cytochalasin D, and 0.01 μM for jasplakinolide, below which the drugs do not cause measurable effects on cell elasticity. However, for a given drug at a given concentration the time between addition of the drug and its effect on cell elasticity varied irregularly between 20 and 120 min, even when the drug was added by exchanging the medium instead of adding a droplet of the drug stock solution. An explanation may be the diffusion and/or convection necessary to deliver the drug to the investigated cell. Even when the medium is exchanged after recording the reference force map, a small meniscus of drug-free medium will remain between the AFM fluid cell and the bottom of the petri dish; thus, the process is still limited by diffusion and convection. Time constants have been estimated to 80 min for diffusion from a droplet of stock solution and as fast as 5 to 10 min for convection (Rotsch et al., 1997a). Convection, however, will strongly depend on, e.g., the temperature gradient between the laboratory and the gas-tight chamber covering our setup and may thus vary strongly from day to day.

Microtubules do not significantly influence cell rigidity

Colchicine and colcemide are agents capable of inhibiting mitosis by sequestering single tubulin molecules (Inoué, 1981; Salmon et al., 1984). On the contrary, taxol binds strongly to intact microtubules, thus stabilizing them and inducing tubulin polymerization (Manfredi et al., 1982; Parness and Horwitz, 1981).

In contrast to the previously discussed experiments we did not measure changes in the cell's elastic properties caused by treatment with drugs affecting the integrity of the microtubule network. In particular, treatment with colchicine and colcemide, which disassemble microtubules similarly to the effect of the cytochalasins on the actin network, did not lead to a softening of the cell. This can be explained by the morphology of the microtubule network. Microtubuli are polar structures. The more slowly growing minus ends originate from the centrosome, an organelle that is typically located close to the cell nucleus. Their plus ends point to the periphery of the cell (Alberts et al., 1997). Unlike stress fibers, microtubuli do typically not extend along the cell periphery, thereby supporting the apical membrane. Whereas the actin network is distributed homogeneously throughout the cell with a pronounced concentration in active regions (Stossel, 1993), microtubules are concentrated in the perinuclear region. Thus, the indentation depth of the AFM tip into the cell (260 nm at 10 kPa and 0.4 nN) may not be sufficient to sense the influence of microtubules on the elastic properties of the investigated cells. Analogously, chemical stabilization of microtubules by means of taxol did not yield any effect on the measured Young's moduli. However, one could expect microtubules to have a stronger influence on elasticity in other cells or under different conditions, e.g., in axons of nerve cells or during mitosis.

Summary

The correlation of AFM data and fluorescence images helped us to better understand and interpret AFM images of living cells. In particular, the frequently observed fibrous structures were identified as stress fibers.

Furthermore, we investigated the effects on cell elasticity of various drugs that disrupt or stabilize the actin or microtubule networks, respectively. Generally, disaggregation of f-actin resulted in a loss of cell rigidity, whereas treatment with drugs like colchicine, colcemide, or taxol that affect microtubules yielded no effect on elasticity. Therefore, we conclude that the actin network mainly determines the elastic properties of living cells.

For the f-actin-disassembling drugs we were able to observe different mechanisms of drug action. An overview of the measured drug effects is given in Table 1. At working concentrations of 10 μM for the cytochalasins and 0.1 μM for Latrunculin A and jasplakinolide we measured reductions in the cell's average Young's modulus by a factor of about three. Although concentration dependencies were hard to quantify, we found all drugs took effect much faster at higher concentrations. In contrast to the other drugs, jasplakinolide did not disrupt stress fibers. Cytochalasin D treatment led to an aggregation of f-actin into felt-like clusters within the cytosol.

The mechanism of cell migration is not yet understood in all details (Lee et al., 1993; Stossel, 1993). However, actin dynamics are supposed to play an important role in it. We could show that actin filaments and their state of polymerization have strong influence on the elasticity of cells. Therefore, elasticity measurements on living cells provide a powerful tool for investigating the mechanisms of the locomotion of animal cells.

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