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The temperature dependence of cell mechanics measured by atomic force microscopy

R Sunyer^{1,2,3}, X Trepap^{1,2,3,5}, J J Fredberg⁵, R Farré^{1,3,4}, and D Navajas^{1,2,3}

D Navajas: dnavajas@ub.edu

¹Unitat de Biofísica i Bioenginyeria, Universitat de Barcelona, 08036 Barcelona, Spain

²Institut de Bioenginyeria de Catalunya, 08028 Barcelona, Spain

³CIBER Enfermedades Respiratorias, 07110 Bunyola, Spain

⁴Institut d'Investigacions Biomèdiques August Pi i Sunyer, 08036 Barcelona, Spain

⁵Program in Molecular and Integrative Physiological Sciences, Harvard School of Public Health, Boston, MA, USA

Abstract

The cytoskeleton is a complex polymer network that regulates the structural stability of living cells. Although the cytoskeleton plays a key role in many important cell functions, the mechanisms that regulate its mechanical behaviour are poorly understood. Potential mechanisms include the entropic elasticity of cytoskeletal filaments, glassy-like inelastic rearrangements of cross-linking proteins and the activity of contractile molecular motors that sets the tensional stress (prestress) borne by the cytoskeleton filaments. The contribution of these mechanisms can be assessed by studying how cell mechanics depends on temperature. The aim of this work was to elucidate the effect of temperature on cell mechanics using atomic force microscopy. We measured the complex shear modulus (G^*) of human alveolar epithelial cells over a wide frequency range (0.1–25.6 Hz) at different temperatures (13–37 °C). In addition, we probed cell prestress by mapping the contractile forces that cells exert on the substrate by means of traction microscopy. To assess the role of actomyosin contraction in the temperature-induced changes in G^* and cell prestress, we inhibited the Rho kinase pathway of the myosin light chain phosphorylation with Y-27632. Our results show that with increasing temperature, cells become stiffer and more solid-like. Cell prestress also increases with temperature. Inhibiting actomyosin contraction attenuated the temperature dependence of G^* and prestress. We conclude that the dependence of cell mechanics with temperature is dominated by the contractile activity of molecular motors.

1. Introduction

Knowledge of the physical mechanisms responsible for the mechanical integrity of living cells is crucial to understanding fundamental cell functions such as differentiation, apoptosis, locomotion and cell growth [1]. The mechanical behaviour of the living cell is largely determined by the cytoskeleton, a tensed polymer network that is able to deform, remodel and flow [2, 3]. This ability is particularly important in lung cells that need to maintain their mechanical integrity while being subjected to cyclic forces owing to breathing [4].

Early studies of cell mechanics revealed that cells are viscoelastic, i.e. they exhibit both solid- and fluid-like features [5, 6]. A robust approach to study such features is to measure the cell complex shear modulus (G^*) using low-amplitude oscillatory measurements over a wide frequency range [7]. In studies of oscillatory mechanics, G^* of cells has been probed

with a variety of techniques such as magnetic twisting cytometry (MTC) [5], optical tweezers [8], microplates [9] and atomic force microscopy (AFM) [6]. All these techniques revealed that the cytoskeleton of a wide variety of cell types exhibits a rheological response that increases with frequency (f) as a weak power law ($\propto f^\beta$, $\beta \sim 0.2$) [5, 6]. In addition, the power-law exponent typically decreases with increasing G' (the real part of G^*) [5]. While these empiric features of cell rheology are well established, the underlying mechanisms are poorly understood.

Mechanisms that could underlie cell rheology include entropic elasticity of cytoskeletal filaments, glassy-like inelastic rearrangements of cross-linking proteins [10, 11] and activity of contractile mechanochemical motors that sets the tensional stress (prestress) borne by the cytoskeletal filaments [12, 13]. Given that each of these mechanisms has a defined dependence on temperature, their contribution to cell mechanical behaviour can be assessed by studying how cell mechanics depends on temperature. This dependence, however, remains largely unknown.

The aim of this work was to assess the temperature dependence of cell mechanics to gain insight into the mechanisms that govern the mechanical behaviour of the cell. We probed the rheology of alveolar epithelial cells at various temperatures by measuring G^* with AFM over a wide frequency range. In addition, we probed cell prestress (P) by mapping the contractile forces that cells exert on their substrates by means of traction microscopy (TM). To assess the role of actomyosin contraction in the temperature-induced changes of G^* and P , we inhibited the Rho kinase pathway of the myosin light chain (MLC) phosphorylation with Y-27632. Our data show that with increasing temperature, cells become stiffer and more solid-like. In addition, cell prestress also increases with temperature. Inhibiting actomyosin contraction attenuated the temperature dependence of G^* and P . Our results show that the dependence of cell mechanics on temperature is dominated by the regulation of the contractile activity of molecular motors.

2. Methods

2.1. Cell culture

The study was carried out on human alveolar epithelial cells A549 (line CCL-185 ATCC, Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 1 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 mg ml⁻¹ streptomycin and 2 µg ml⁻¹ amphotericin B (all from GIBCO, Gaithersburg, MD), 10% inactivated foetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) and buffered with HEPES (Sigma, St Louis, MO). Cells were incubated at 37 °C and 5% CO₂. Two days before the experiments, cells were harvested by means of a brief exposure to trypsin EDTA and plated sparsely on 25 mm diameter glass coverslips (900 cells mm⁻²) for AFM experiments or polyacrylamide gels (~30 cells mm⁻²) for TM experiments. One day before experiments, cells were serum-deprived to arrest the cell cycle in the G₁/G₀ phases.

2.2. Atomic force microscopy measurements

2.2.1. Measurements—Cell rheology was probed with a custom-built AFM attached to an inverted optical microscope (Zeiss Axiovert S 100, Oberkochen, Germany) equipped with a closed-loop temperature-controlled microincubator (HCMIS ALA Science, NY) by using a previously described method [6]. Cells were indented with V-shaped silicon nitride cantilevers (nominal spring constant $k = 0.01$ N m⁻¹) with a spherical polystyrene bead of radius $R = 2.25$ µm glued at their end (Novascan Technologies, Ames, IA). The actual spring constant of the cantilever was calibrated by using the thermal fluctuations method [14, 15]. The relationship between the photodiode signal and the cantilever deflection (d) was

computed as the slope of the force–displacement curve obtained on a bare region of the coverslip. The force (F) on the cantilever was computed using Hooke's law ($F = kd$). Cell rheology was probed at the nuclear region as determined by bright field microscopy. For each cell, we acquired five force-displacement curves (F – z , z being the displacement of the piezotranslator) while the piezotranslator was ramped forward and backward at constant speed (1 Hz, 5 μm amplitude, $\sim 1 \mu\text{m}$ maximum indentation). From the curve, we determined the zero force offset and the contact point (F_0 , z_0) between the cell and the cantilever tip. Cell indentation (δ) was computed as $\delta = z - z_0 - d$. Subsequently, the tip was placed at an operating indentation (δ_0) of $\sim 600 \text{ nm}$, and a low-amplitude (50 nm) multi-frequency oscillation composed of five sine waves (0.1, 0.4, 1.6, 6.4 and 25.6 Hz) was applied for 120 s [16]. The indentation depth used was high enough to prevent the uncertainty in $G^*(f)$ near the contact point but low enough to ensure that measurements were done in the range where the Hertz contact model and its expansion to small oscillations are valid [17]. Finally, the contact point was again determined to account for possible drifts. To correct for the hydrodynamic viscous drag, we measured the drag force on the cantilever (F_d) by recording three F – z curves (0.1 Hz, 5 μm amplitude) with a superimposed 102.4 Hz sinusoidal oscillation of 50 nm amplitude [18]. The drag factor $b(h)$ was computed at different tip–substrate separations (h) as $b(h) = F_d/v$, v being the relative cantilever-liquid velocity ($v = \dot{d} - \dot{z}$, where dot indicates the time derivative). The drag factor at contact ($b(0)$) was determined by extrapolating $b(h)$ to $h = 0$ with a scaled spherical hydrodynamic model [18]. $G^*(f)$ was measured in $n = 6$ samples (~ 6 cells per sample) for each temperature (selected in random order). To assess the intra-cellular variability of the temperature response, we obtained paired measurements of $G^*(f)$ at the nuclear and peripheral cell regions (selected in random order) in $n = 6$ samples (~ 6 cells per sample) for each temperature. The role of the Rho kinase signalling pathway of MLC phosphorylation was assessed by pretreating six cell samples with Y-27632 (10 μM), 30 min before measurements.

2.2.2. Data processing— G^* was computed from the AFM oscillatory measurements as described previously [6,17]. The force applied on the cantilever was corrected for the zero-force offset that was computed as the mean force of the noncontact part of the F – z curve recorded on each cell. Force-indentation data were analysed with the spherical Hertz model [19]:

$$F = \frac{4E\sqrt{R}}{3(1-\nu^2)}\delta^{3/2}, \quad (1)$$

where E is Young's modulus and ν is Poisson's ratio of the cell (assumed to be 0.5). Substituting $F = kd$ and $\delta = z - z_0 - d$ into equation (1),

$$d = \frac{4E\sqrt{R}}{3(1-\nu^2)k}(z - z_0 - d)^{3/2}.$$

A nonlinear least-squares fit (Matlab 6.5, The MathWorks, Inc., Natick, MA) was used to estimate E and z_0 from each loading d – z curve. The fit included a clear noncontact region ($> 1 \mu\text{m}$).

For low-amplitude oscillations around an operating indentation, equation (1) can be approximated by taking the first term of the Taylor expansion [20]:

$$F - F_0 \approx \left(\frac{2E\sqrt{\delta_0 R}}{1-\nu^2}\right)(\delta - \delta_0). \quad (2)$$

Expressing equation (2) in terms of the shear modulus $G = E/2(1 + \nu)$ and solving it for G ,

$$G = \frac{1 - \nu}{4\sqrt{\delta_0 R}} \left(\frac{F - F_0}{\delta - \delta_0} \right).$$

This approximation can be expressed in the frequency domain through the elastic–viscoelastic correspondence principle [6] leading to

$$G^*(f) = \frac{1 - \nu}{4\sqrt{\delta_0 R}} \left(\frac{F(f)}{\delta(f)} - i2\pi b(0)f \right),$$

where $i2\pi b(0)f$ is the correction for the viscous drag. G^* was separated into its real and imaginary parts $G^* = G' + iG''$, with G' and G'' being the elastic and loss moduli, respectively. G' is a measure of the elastic energy stored and recovered per cycle of oscillation whereas G'' accounts for energy dissipation due to internal friction.

2.2.3. Modelling— G^* data were fitted with the power-law structural damping model [5]

$$G^*(f) = G_0(1 - i\eta)(f/f_0)^\beta + i2\pi f\mu, \quad (3)$$

where β is the power-law exponent, G_0 is a scale factor for the storage and loss moduli, f_0 is a frequency scale factor that for simplicity was taken to be 1 s^{-1} , μ is a Newtonian viscous term and $\eta = \tan \pi\beta/2$ is the hysteresivity coefficient which is an index of the solid-like ($\ll 1$) or liquid-like ($\gg 1$) behaviour of the cell. The three independent parameters of the model (G_0 , β and μ) were obtained by fitting equation (3) to the average G^* by nonlinear regression analysis (SigmaPlot, SPSS, Chicago, IL).

2.3. Traction microscopy measurements

2.3.1. Polyacrylamide gels—Preparation of thin collagen-coated polyacrylamide gel disks was carried out as described previously [21, 22]. Green fluorescent latex beads of 200 nm in diameter (Invitrogen, Carlsbad, CA) were mixed with a 2% acrylamide and 0.3% bis-acrylamide solution (Bio-Rad, Richmond, CA) (1:125 vol vol⁻¹ bead solution volume of acrylamide mixture). To polymerize the solution, we added 10% of ammonium persulfate and subsequently TEMED (both purchased from Sigma, St Louis, MO). A volume of 10 μl of the resulting polyacrylamide solution was immediately placed onto a glass coverslip of 25 mm in diameter. Then, a coverslip of 12 mm in diameter was placed on top of the polyacrylamide solution. After 45 min, the solution was completely polymerized and the 12 mm coverslip was carefully peeled off. Type I collagen (200 mg ml⁻¹) was added to the gel surface for 2 h to coat the gel.

2.3.2. Measurement of gel stiffness at different temperatures—Young's modulus of the polyacrylamide gel at different temperatures was measured with atomic force microscopy. Gels were probed with a V-shaped cantilever (spherical tip of radius $R = 2.25 \mu\text{m}$, nominal spring constant of $k = 0.01 \text{ nN m}^{-1}$; Novascan, Ames, IA). After AFM calibration, F - z curves (1 Hz, 5 μm amplitude, $\sim 1 \mu\text{m}$ maximum indentation) were obtained over 6–10 separated gel regions. Young's modulus of the gel was estimated by fitting indentation data with the Hertz spherical model (equation (1)) using nonlinear least-squares regression. We performed this protocol for $n = 5$ gel samples at 13 and 37 °C. E was $277 \pm 14 \text{ Pa}$ and $271 \pm 25 \text{ Pa}$ (mean $\pm$ SE) at 13 and 37 °C, respectively. This small difference was

not statistically significant (paired t -test, $p = 0.706$). Therefore, the average E measured in the gels was used for computing cell traction forces from bead displacement measurements.

2.3.3. Optical microscopy—Coverslips containing cell-cultured polyacrylamide gels were mounted on the stage of an inverted fluorescence microscope (Eclipse TE2000, Nikon) equipped with a closed-loop temperature-controlled microincubator (HCMIS ALA Science, NY) and placed on a vibration isolation table (Isostation, Newport, Irvine, CA). Images were acquired using a long working distance objective (40 $\times$) and a 12 bit resolution cooled CCD camera (Orca AG, Hamamatsu Photonics, Japan). The apparent pixel size after magnification was 0.157 μm with a field of view of $150 \times 150 \mu\text{m}^2$.

2.3.4. Measurements—A bright field image of an isolated cell was captured to determine its boundary at a randomly chosen temperature (13 or 37 $^{\circ}\text{C}$). Subsequently, the apical surface of the gel was focused, and a fluorescence image of the beads embedded near the apical surface of the gel was acquired. Then, the sample was cooled or heated at 2 $^{\circ}\text{C min}^{-1}$ until it reached the other temperature (37 or 13 $^{\circ}\text{C}$) and new bright field and fluorescent images were recorded. At the end of the experiment, trypsin was added to remove the cells from the polyacrylamide gel. Finally, an additional fluorescent image was recorded at room temperature to determine the position of the beads in the unstrained gel (reference image). Measurements were obtained in $n = 11$ samples (one cell per sample). Finally, we studied cells pretreated with Y-27632 (10 μM) for 30 min ($n = 9$ samples, 1 cell per sample).

2.3.5. Data processing—The displacement field between each fluorescence image and the corresponding reference image was computed using the image correlation method [23]. Images were iteratively divided into smaller window areas, and 2D cross-correlation was calculated between a pair of images. The location of the cross-correlation peak was taken as the displacement vector of the window. The cell boundary was drawn manually from the bright field image. The traction field ($\mathbf{T} = T_f(x, y)$) was computed with a spatial resolution of 2.5 μm from the displacement field by using constrained Fourier transform traction cytometry [24] and Young's modulus of the gel. For each traction field, the net contractile moment (M) was computed as defined by Butler and coworkers [24]. M is a measure of the cell contractile strength [25]. The cell prestress was computed as described by Wang and coworkers [25]. Briefly, P was obtained as

$$P = \frac{1}{A''} \int_{A'} \mathbf{T} \cdot \mathbf{n} dA, \quad (4)$$

where A'' and A' are the projected and the cross-sectional areas of the cell, respectively, and $\mathbf{n}$ is the unit vector normal to A' . For each cell and temperature, P was calculated for different cell cross sections, and the average value was obtained.

2.4. Statistical analysis

Data are shown as mean $\pm$ SE. The effect of temperature on G^* and the power-law exponent β were analysed by two-way and one-way analyses of variable (ANOVA), respectively. Comparison between nuclear and peripheral cell regions was analysed by a paired t -test. The effect of temperature on M and P was analysed by a paired t -test.

3. Results

An example of force-indentation ($F-\delta$) curve recorded in an A549 cell is shown in figure 1. For $\delta < 0$, the cantilever was not in contact with the sample and the force was zero. When the cantilever contacted the sample ($\delta > 0$, solid line) the force beyond the contact point

(arrow) exhibited a nonlinear shape due to the increase in contact area as the spherical cantilever tip indented the sample. The withdrawing curve (dashed line) displayed hysteresis revealing viscoelastic behaviour. The negative forces of the withdrawing curve at low indentations indicate unspecific adhesion. The small separation between the forward and the withdrawing curve in the non-contact region was caused by hydrodynamic viscous drag. The dotted line indicates operating force (F_0) and indentation (δ_0) at which oscillatory indentations were applied.

$G^*(f)$ measurements at the nuclear region of the cell were taken at 37, 21 and 13 °C with operating indentations of 537 ± 43 nm, 597 ± 83 nm and 617 ± 39 nm, respectively. At physiological temperature (37 °C), the storage and loss moduli at 0.1 Hz were $G' = 300 \pm 68$ Pa and $G'' = 95 \pm 14$ Pa, respectively. As temperature decreased to 13 °C, G' and G'' experienced a ~ 1.5-fold fall ($p < 0.001$ for G' and $p = 0.259$ for G'') (figures 2(A) and (B)). Inter-cellular variability as measured by the coefficient of variation (CV = SD/mean) was 84% at 37 °C and decreased to 48% at 13 °C. At 37 °C, the frequency dependence of G' and G'' exhibited power-law behaviour with a value of G''/G' that was nearly constant for the lower frequencies indicative of a coupling between elastic and dissipative stresses. The power-law behaviour was maintained at lower temperatures. The power-law model (equation (3)) described G^* data very well ($r^2 > 0.97$) for each temperature (table 1 and figure 2). The stiffness scale factor G_0 showed a 1.7-fold rise as temperature increased from 13 °C to 37 °C. Cell stiffening was associated with a 40% fall ($p < 0.05$) in the power-law slope β and a similar decrease in hysteresivity (η) indicating more solid-like behaviour. The Newtonian viscous term μ was two-fold higher at 13 °C than at 37 °C.

To assess intra-cellular variability, we measured the temperature dependence of $G^*(f)$ at the nuclear and peripheral cell regions (figure 3). At every temperature measured, the cell periphery was stiffer than the nucleus ($p < 0.05$ at 37 °C and $p < 0.01$ at 13 °C). The dependence of $G^*(f)$ on temperature was qualitatively similar in both cellular regions but more pronounced at the cell periphery. As in the nuclear region, $G^*(f)$ at the periphery exhibited power-law behaviour with a decrease of 40% ($p < 0.05$) in the power-law exponent as the temperature decreased from 37 °C and 13 °C (table 2 and figure 3).

Inhibiting the Rho kinase pathway preserved the power-law behaviour with a reduction of the baseline value of G_0 and an increase in the power-law slope, indicating more fluid-like behaviour with respect to the untreated cells (table 1). As temperature decreased to 13 °C, G' and G'' at 0.1 Hz experienced a non-significant ~ 15% fall ($p = 0.157$) and ~7% fall ($p = 0.259$), respectively (figures 2(C) and (D)).

Cell contraction exhibited a temperature dependence parallel to that of G^* . At physiological temperature, cells showed traction forces predominantly located along cell periphery and pointing to the cell centre (figure 4). As temperature decreased to 13 °C, the pattern of traction forces was maintained but with lower force magnitude. Cell spreading area changed little with temperature (9% increase from 37 °C to 13 °C) suggesting that the decrease of traction observed after reducing temperature was not due to a partial cell detachment. The net contractile moment of the cells at 37 °C was 0.25 ± 0.06 pNm and decreased ~ 1.5-fold ($p < 0.01$) as temperature was reduced to 13 °C (figure 5(A)). Similar temperature dependence was observed when measuring prestress (figure 5(B)). At physiological temperature, P was 92 ± 11 Pa and decreased ~two-fold when temperature fell to 13 °C. Pretreatment with Y-27632 decreased baseline contraction (figures 5(C) and (D)). As temperature was reduced to 13 °C, M decreased by 32% ($p < 0.05$) and P decreased by ~15% although this latter difference did not reach statistical significance ($p = 0.578$).

4. Discussion

We used AFM to measure the temperature dependence of the complex shear modulus of alveolar epithelial cells over a wide frequency range. At each temperature tested, G^* was described by scale-free behaviour which increased with frequency as a weak power law and showed coupling between elastic and loss moduli. At physiological temperature, G was higher than at 13 °C at both the nuclear and the peripheral cell regions. This temperature-induced stiffening was associated with more solid-like behaviour and a reduction in the power-law exponent. TM experiments revealed a temperature dependence of cell prestress similar to that of G . Reduction of MLC phosphorylation with Y-27632 attenuated both the basal tone of the cell and the temperature-induced response of G^* and prestress.

The effect of temperature on cell stiffness has been previously studied using magnetic twisting cytometry [26–28]. These studies reported that increasing temperature induced a decrease in cell stiffness [26, 27] and an increase in β [28]. The discrepancy of such findings with those reported here could arise from the fundamental differences between MTC and AFM. MTC probes cell stiffness by binding magnetic microbeads (~5 μm in diameter) to the cytoskeleton via cell surface receptors, oscillating the beads horizontally with an external magnetic field and measuring the resulting bead rotation [29]. The change in cell stiffness is defined as the ratio of the applied torque to the resulting bead displacement. While MTC offers the advantage of a direct connection of the probe to the cytoskeleton, it also requires attachment of microbeads to the actin cytoskeleton through focal contacts, which results in partial microbead embedding and local remodelling of the actin meshwork [30]. The effect of temperature on focal adhesion assembly, bead embedding and local actin remodelling remains largely unknown. However, spontaneous motion of microbeads attached to cells varies dramatically with temperature, thereby suggesting that large changes may occur in the local environment of the bead [28]. Such changes could bias the temperature dependence of $G^*(f)$ measured with MTC. AFM overcomes these limitations and allows the measurement of G^* with minimal cell perturbation by indenting the cell surface with the cantilever tip [6]. The use of silicon nitride cantilevers with a microsphere at its end served various purposes. First, the spring constant of silicon nitride cantilevers is stable in the temperature range used in our study [31, 32]. Second, the use of microspheres instead of the more common pyramidal tips allowed us to indent the cells with a well-defined tip-cell contact geometry [16, 17]. Additionally, the large diameter of the microspheres (4.5 μm) allowed us to test a cell area (~4 μm^2) larger than the cytoskeleton mesh size, thereby providing a local average of the cell's shear modulus. We used an operating indentation of ~600 nm with a superimposed multi-frequency oscillation of 50 nm amplitude. This indentation depth was high enough to overcome the uncertainty in G^* for indentations < 200 nm and low enough (<20% of cell thickness) to avoid hardening effects due to the underlying hard substrate [17]. Finally, the multi-frequency AFM oscillation minimizes the problems associated with cantilever drift and cell movements, providing a simultaneous measure of G^* over a wide frequency range.

We found that the cell peripheral region was stiffer than the nuclear region at both 13 and 37 °C. Similar results had been previously reported in other cell types supporting the generality of this finding [33, 34]. Changes with temperature at the peripheral region were qualitatively similar but quantitatively more pronounced than those at the nuclear region. This difference in temperature sensitivity could be explained by the different mechanical properties of the structures that bear mechanical stresses in both cellular regions. While at the cell periphery stresses are largely borne by the actomyosin CSK, stresses at the nuclear region are likely to be borne not only by the actomyosin CSK but also by the nucleus. We thus speculate that nuclear mechanics might be less sensitive to temperature changes than the actomyosin CSK. Further studies should address this issue in greater depth.

Compared with MTC or AFM, TM is an independent method of measurement that does not require application of a probe to the cell apical surface. TM provides a measure of cell prestress, which is indicative of cell stiffness [13, 25, 35]. Using this technique, we measured temperature-induced changes in cell prestress from the map of traction forces exerted by the cell onto the gel. TM uses elastic soft substrates with embedded fluorescent beads ($\sim 0.2 \mu\text{m}$ in diameter, figure 4(B)). Under cell contraction, these substrates deform without forming wrinkles. Traction maps are computed by comparing deformed with relaxed gel substrates obtained before and after removing the cells with trypsin (see section 2). The stiffness of the gel ($\sim 300 \text{ Pa}$) was adjusted to maximize the signal-to-noise ratio of gel displacements. The use of a polyacrylamide gel as a soft substrate ensures stable gel stiffness within the temperature range of our experiments. From 37 to 13 °C, Young's modulus of the gel experienced a non-significant 2% decrease. Such small variation indicates that temperature-induced changes in the entropic elasticity of flexible polymer gels are negligible in the temperature range of our experiments. We computed the mean prestress by balancing the traction exerted by cells to their underlying substrate with cell internal stresses. It should be noted that P represents the portion of the prestress balanced by the traction at the interface between the cell and its substrate. This portion represents the main contribution to prestress since compression-supporting structures (such as microtubules) have been reported to contribute only a 14% on average [36].

Scale-free rheology with G^* increasing with frequency as a weak power law has been reported from MTC and AFM measurements obtained on various cell types at room temperature [5, 6, 12, 37]. Our measurements of G^* obtained from 37 to 13 °C show that the power-law behaviour of the cell prevails over a wide temperature range. In addition, our findings provide further support to the notion that the generalized Stokes–Einstein relationship (GSER) is not applicable in cells [28]. Indeed, observations of passive tracers attached to the cytoskeleton network revealed a dramatic decrease of the tracer's fluctuations when temperature was decreased from 37 to 12 °C [28, 38]. The application of the GSER to these data would imply that G^* increased by $\sim$ two orders of magnitude with decreasing temperature, which is clearly inconsistent with our results.

Various mechanisms with different temperature dependences may contribute to cell rheology. One of such mechanisms is the entropic elasticity of the semiflexible polymer network. Entropic elasticity arises from the end-to-end distance reduction of individual filaments caused by thermally driven transverse fluctuations. Each filament acts as an entropic spring and its behaviour is described by the worm-like chain model, which also describes other biopolymers such as DNA [39]. Such entropic contribution predicts a linear increase in the network elastic modulus with temperature [40]. In the temperature regime used in our study, the expected change in the elastic modulus due to entropic elasticity is less than 10%. This change is much smaller than that we observed, indicating that the entropic contribution of semiflexible polymers must be small compared with other mechanisms. This finding is consistent with the estimation obtained by Fernandez *et al* using a completely different approach [13]. A second mechanism contributing to cell rheology originates in the localized rearrangements of cytoskeleton cross-links. It has been proposed that the deformation of the cytoskeleton is associated with thermally activated unbinding and unfolding of cytoskeletal protein domains [11]. These molecular rearrangements could be at the origin of the glassy-like behaviour of the living cell [41]. Molecular rearrangements of this kind can be described by the Eyring equation which relates the macroscopic flow ($\dot{\gamma}$) to the applied stress (σ) as $\dot{\gamma} = \sigma \exp(-\Delta H/k_B T)$, where ΔH is the potential energy and k_B is the Boltzman constant [7]. In glasses, this kind of structural rearrangements leads to a strong dependence of viscosity on temperature [42]. Estimations of activation energies of unfolding events can be obtained from single molecule experiments and give energy barriers in the range of $\Delta H = 10\text{--}60 k_B T$ [43, 44]. Given the exponential

dependence of molecular rearrangements with inverse temperature, the loss modulus G'' of the cell should decrease dramatically as temperature is reduced from 37 to 13 °C. Our data are in contrast to this prediction, indicating that changes induced by temperature on cytoskeleton bonds and cross-links are compensated by an additional mechanism.

Such a mechanism is likely to be a change in the contractile activity of molecular motors. Indeed, we observed that increasing temperature enhances cell contraction, as indicated by the 1.5-fold reduction of the net contractile moment. Interestingly, reducing the actomyosin activity by inhibiting MLC phosphorylation [45] attenuated the baseline of G^* and P and their temperature dependence. Moreover, a temperature-induced enhancement of actomyosin activity is consistent with previous observations that temperature increases contractility in fibroblasts [46] and activates the actomyosin machinery [47–52]. Such an increase has been attributed either to an enhancement of the mean force developed by the individual actomyosin cross bridges [50–52] or to a change in the cross-bridge number [47, 49]. The parallel behaviour of G' and P observed here indicates that the link between both magnitudes is maintained at different temperatures. This finding is not at all obvious given the wide diversity of responses that actin networks exhibit with small changes in temperature [53]. Our observations point to a picture of the cytoskeleton where the contractility of mechanochemical motors can overwhelm other contributions to cell elasticity to provide an integrated mechanical response.

5. Conclusion

To investigate the different mechanisms that contribute to cell rheology, we explored the temperature dependence of the mechanical response of living cells using AFM and TM. We showed that with increasing temperature, cells become stiffer and more solid-like. This change was tied to a rise in the contractile tone of the cell resulting in an increase of prestress. In addition, inhibiting MLC phosphorylation attenuated the baseline values of G^* and of cell prestress and their temperature dependence. Our data suggest that temperature induces an increase in the efficiency or in the number of the molecular motors inside the cell, which modifies cytoskeletal internal tension and cell stiffness. This mechanism overwhelms the entropic contribution of the semiflexible polymer network and the contribution of the glassy-like inelastic rearrangements of cross-linking proteins. Our observations support the notion that the contractile mechanochemical motors are key elements that allow cells to modulate their rheology without varying the polymerization, the density or the cross-linking state of cytoskeletal filaments.

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Figure 1.

Atomic force microscopy measurements. (A) Illustration of an AFM cantilever with a spherical tip probing cell rheology. (B) Force-indentation (F - δ) curve obtained as the cantilever tip approached an A549 cell (thick line) at $10 \mu\text{m s}^{-1}$ and then retracted (thin line) at the same speed. The retraction curve shows adhesion at low indentations (forces below the retraction plateau). The forward curve was used to determine the contact point between the tip and the sample (arrow). Starting from the arrow, positive values of δ indicate indentation while negative values indicate the tip-sample distance. Once the contact point was determined, the cantilever was placed at an operating indentation (δ_0) and a multi-frequency signal of 50 nm amplitude was applied to measure the complex shear elastic modulus of the cell. F_0 is the operating force of oscillatory measurements.

Figure 2. Temperature dependence of cell complex elastic modulus (G^*) measured with AFM. Frequency dependence of the storage modulus (G' , real part of G^*) (A) and the loss modulus (G'' , imaginary part of G^*) (B) measured on A549 cells at different temperatures. (C) and (D) Effect of inhibiting Rho kinase (10 μ M Y27632) on the temperature-induced change in G^* . Data are mean $\pm$ SE. Solid lines are the fit of the power-law structural damping model (equation (3)). The parameters of the fit are shown in table 1.

Figure 3.

Complex elastic modulus (G^*) measured at the nuclear and the peripheral cell regions with AFM. Frequency dependence of the storage modulus (G' , real part of G^*) (A) and the loss modulus (G'' , imaginary part of G^*) (B) measured on A549 cells at 13 °C for the nuclear and the peripheral cell regions. (C) and (D) Frequency dependence of G' and G'' at 37 °C for the central and the peripheral cell regions. Data are mean $\pm$ SE. Solid lines are the fit of the power-law structural damping model (equation (3)). The parameters of the fit are shown in table 2.

Figure 4.

Temperature-induced changes in cell contraction measured with traction microscopy. (A) Bright-field image of an A549 cell plated on a polyacrylamide gel with embedded fluorescent microbeads. (B) Fluorescence image of the microbeads below the gel surface. (C), (D) Traction fields of the cell at 37 and 13 °C. Cell boundary drawn from the bright-field image is shown as a white line. The colour scale indicates the magnitude of traction force (in Pa). The arrows depict the direction and relative magnitude of traction force. Traction fields were calculated with 2.5 μm spatial resolution. The scale bar is 10 μm .

Figure 5. Effect of temperature on cell contraction. (A) Net contractile moment (M). (B) Cell prestress. (C) and (D) Effect of inhibiting Rho kinase (10 μ M Y-27632) on the temperature-induced change in M and P . Data are mean $\pm$ SE. * is $p < 0.05$ (paired t -test).

Table 1

Parameters of the power-law structural-damping model fitted at different temperatures and treatments.

Temperature	Treatment	G_0 (Pa)	β	μ (Pa s)	r^2
13 °C	–	193 ± 20	0.192 ± 0.025	3.424 ± 0.282	0.976
21 °C		189 ± 11	0.180 ± 0.014	2.264 ± 0.147	0.989
37 °C		326 ± 10	0.137 ± 0.008	1.640 ± 0.126	0.994
13 °C	Y-27632	107 ± 0.5	0.261 ± 0.001	0.606 ± 0.009	0.999
37 °C		145 ± 10	0.241 ± 0.011	1.55 ± 0.126	0.991

Table 2

Parameters of the power-law structural-damping model fitted at different temperatures and cell regions.

Temperature	Cell region	G_0 (Pa)	β	μ (Pa s)	r^2
13 °C	Nuclear	215 ± 2	0.236 ± 0.003	1.320 ± 0.039	0.9998
37 °C		366 ± 6	0.163 ± 0.037	0.722 ± 0.073	0.9995
13 °C	Periphery	303 ± 5	0.256 ± 0.040	2.294 ± 0.089	0.9997
37 °C		659 ± 11	0.188 ± 0.039	2.226 ± 0.148	0.9995