Effects of microtubules and microfilaments on \([\text{Ca}^{2+}]_i\) and contractility in a reconstituted fibroblast fiber

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Obara, Kazuo, Koji Nobe, Hiromi Nobe, Michael S. Kolodney, Primal de Lanerolle, and Richard J. Paul. Effects of microtubules and microfilaments on \([\text{Ca}^{2+}]_i\), and contractility in a reconstituted fibroblast fiber. Am J Physiol Cell Physiol 279: C785–C796, 2000.—We used a reconstituted fiber formed when 3T3 fibroblasts are grown in collagen to characterize nonmuscle contractility and \([\text{Ca}^{2+}]_i\) signaling. Calf serum (CS) and thrombin elicited reversible contractions repeatable for >8 h. CS elicited dose-dependent increases in isometric force; 30% produced the largest forces of 106 ± 12 μN (n = 30), which is estimated to be 0.5 mN/mm² cell cross-sectional area. Half times for contraction and relaxation were 4.7 ± 0.3 and 3.1 ± 0.3 min at 37°C. With imposition of constant shortening velocities, force declined with time, yielding time-dependent force-velocity relations. Forces at 5 s fit the hyperbolic Hill equation; maximum velocity (\(V_{\text{max}}\)) was 0.035 ± 0.002 L/s. Compliance averaged 0.0076 ± 0.0006 L/Fo. Disruption of microtubules with nocodazole in a CS-contracted fiber had no net effects on force, \(V_{\text{max}}\), or stiffness; force increased in 8, but decreased in 13, fibers. Nocodazole did not affect baseline intracellular \([\text{Ca}^{2+}]_i\) concentration (\(\text{[Ca}^{2+}]_i\)) but reduced (∼30%) the \([\text{Ca}^{2+}]_i\) response to CS. The force after nocodazole treatment was the primary determinant of stiffness and \(V_{\text{max}}\), suggesting that microtubules were not a major component of fiber internal mechanical resistance. Cytochalasin D had major inhibitory effects on all contractile parameters measured but little effect on \([\text{Ca}^{2+}]_i\).

cytochalasin D; nocodazole; nonmuscle mechanics; Swiss 3T3; tensegrity; intracellular calcium concentration

THE ROLE OF CYTOSKELETAL FILAMENT networks in modulating nonmuscle contractility is unclear. Giuliano and colleagues (7) have proposed that nonmuscle contractility is regulated both by modulating the activity of molecular motors, such as myosin II, and by altering the cytomatrix in such a manner as to either resist or yield to the tension applied by the motors. This hypothesis is supported by the observation that tension increases in response to disruption of microtubules by nocodazole (5) in cultured fibroblasts (4) and reconstituted fibroblast fibers (21). These data are also consistent with the view that microtubules provide some form of internal resistance. In tensegrity models (6), a portion of a cell’s contractile force is borne by rigid internal structures, reducing the force transmitted to external structures, as monitored by force transducers. Alternatively, other mechanisms may underlie the effects of nocodazole. For example, the increase in smooth muscle contractility elicited by nocodazole has been correlated with an increase in intracellular \([\text{Ca}^{2+}]_i\) concentration (\([\text{Ca}^{2+}]_i\)) (17).

If microtubules constitute an internal resistance in parallel with the actin-myosin network and are capable of bearing compressive forces, depolymerization of microtubules should result in a greater measured force as the load shifts from internal microtubule structures to the external force transducer. One would also expect that other mechanical parameters, such as cell mechanical stiffness and shortening velocity, would be sensitive to the presence of an intracellular mechanical resistance as that postulated for microtubules. In fact, on theoretical grounds, one might anticipate that the maximum shortening velocity (\(V_{\text{max}}\)) would be particularly sensitive to intracellular resistances.

With the use of similar arguments, the effects of microfilament assembly in fibroblast contractility could also be deduced by studying the mechanical effects of cytochalasin D, known to disrupt microfilaments (3).

In this study, we developed a reconstituted fibroblast fiber, based on previous models (19, 21), that enables not only force to be quantitated but also velocity and stiffness in nonmuscle cells. We report that disruption of microfilaments has a profound effect on fibroblast contractility but not \([\text{Ca}^{2+}]_i\). Our detailed mechanical analysis, in contrast to hypotheses based on measurement of isometric force alone, does not support a role for microtubule resistance in the mechanical properties of reconstituted fibroblast fibers.
MATERIALS AND METHODS

Cell culture and reconstitution of fibroblast into fibers. Swiss 3T3 fibroblast cells (passages 15–30) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum (CS) and antibiotic-antimycotic (Ab/Am; 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) at 37°C in a humidified, 5% CO2-95% O2 atmosphere. Fibroblast fibers were formed by growing 3T3 fibroblasts in rat tail collagen (Upstate Biotechnology, Lake Placid, NY) gel matrix by a modification of the method of Kolodney and Wysolmerski (21). Dispersed cells were suspended in an ice-cold collagen solution that contained 2 × 106 cells/ml and 0.5 mg/ml rat tail collagen in DMEM + 10% CS with added Ab/Am (Sigma). An aliquot (2 ml) of the collagen/cell suspension was poured into a well (0.8 × 5 cm × 0.5 cm deep) cut in a layer of silicone rubber in 100-mm glass petri dishes and placed in a CO2 incubator at 37°C. After 8 h, an additional 1 ml of DMEM + 10% CS and Ab/Am were added to each well. The preparations were incubated for 3–5 days or until the cells shrank the gel and formed a fiber. Except where noted, cells were placed in serum-free media the night preceding the day on which experiments were conducted.

Mechanics measurements. The fibers made from 3T3 fibroblasts were cut into 5-mm pieces and mounted between glass posts with a cyanoacrylate glue. One post was fixed and the other connected to an AME 801 silicon strain gage (Sensonor) force transducer. The fibers were bathed in physiological salt solution (MOPS-PSS) that contained (in mM): 140 NaCl, 4.7 KCl, 1.2 NaH2PO4, 0.02 EDTA, 1.2 MgSO4, 2.5 CaCl2, 5.5 dextrose, and 20 MOPS, pH 7.4, at 37°C.

For measurement of shortening velocity and stiffness, fibers (5-mm) were glued between the force transducer and a lever arm of which its position, and therefore the length of fiber, was controlled by a Cambridge Technology (Cambridge, MA) ergometer. Force-velocity relations were measured by imposing constant shortening velocities on the fiber, and measurement of the subsequent force. A series of eight different velocities at 60-s intervals was used for measurement of each individual force-velocity relation. Force-velocity data were fitted with the Hill equation, $F = \frac{F_0}{1 + \left(\frac{V}{V_{max}}\right)^n}$, where $F$ is the force, $F_0$ is the maximum force, $V$ is the velocity, $V_{max}$ is the maximum velocity, and $n$ is the Hill constant. Velocity was measured with an Intracellular Imaging (Cincinnati, OH) microscope-based system (InCa system). This fibroblast chamber was placed on a Nikon Diaphot inverted microscope with a fluophase objective. Fluorescent images of cells excited at 340 and 380 nm and emitted at 510 nm were obtained with a Dage silicon-intensified target camera. After subtraction of background fluorescence, the 340 and 380 nm images were ratioed on a pixel by pixel basis, and the ratios converted to [Ca2+]i using a standard curve. Solutions containing known concentrations of free Ca2+ (Molecular Probes) were used to generate this standard curve. Fluorescence intensity was measured in 150 μl for each standard solution (0, 0.065, 0.100, 0.225, 0.351, and 0.602 μM free Ca2+ concentration) containing 13.3 μg/ml fura 2 pentapotassium salt. Quantitative analysis of the average subcellular Ca2+ was performed by defining the outline of the cell, summing the Ca2+ in all the pixels within the defined area, and dividing by the number of pixels.

Confocal fluorescence microscopy. For phalloidin staining, fibers were fixed with 4% paraformaldehyde and embedded in 17% gelatin. Sections were cut at 100 μm using a Leica VT 1000 vibrating blade microtome and stained with rhodamine-conjugated phalloidin (Sigma, St. Louis, MO). Images were constructed from optical sections acquired by scanning confocal microscopy.

For microtubule staining, fibers were fixed with 4% paraformaldehyde, and 100-μm sections were cut with a Leica VT 1000 vibrating blade microtome. Sections were blocked with 5% goat serum and incubated overnight in a 1:100 dilution of monoclonal antibody to α-tubulin (Amersham) followed by FITC-conjugated goat anti-mouse (Zymed, San Francisco, CA) at 1:50. Images were acquired with an Olympus epifluorescence microscope equipped with a high-resolution charge-coupled device camera.

Data analysis. All data are presented as means ± SE. Control and serum data were pooled and include some data previously reported (23). To assess the effects of cytochalasin D and nocodazole, a control contraction was elicited by 30% serum and the mechanical parameters were measured. After treatment with the drugs, the mechanical parameters were again measured. Paired comparisons, with each fiber serving as its own control, were made to control for variability among fibers. Differences were analyzed with paired t-tests; differences with a P value <0.05 were accepted as statistically significant.

RESULTS

Structure of artificial fibers. The formation of a fiber generally takes 3 days after the cell/collagen suspension is poured into the mold. This transformation is shown in Fig. 1. The final fiber has dimensions of ~40 mm long and 1–2 mm diameter. For contractility measurements, each fiber was cut into smaller segments (~5 mm). These segments are about one-eighth of the whole fiber and would, if proportional, contain ~5 × 105 of the original cells. Histological analysis of sections indicated that the cells were generally aligned along the long axis of the fiber. A more detailed structural analysis of these types of fibers at both aggregate and intracellular levels has been reported (21).

Isometric contraction. Fibers were mounted under isometric conditions, then the length was increased to match the original fiber length in the mold. After stress relaxation, force attained a baseline level of 53.0 ± 0.003 μN (n = 30). Addition of serum increased isometric force that returned to prestimulus levels on washout (Fig. 2). These contraction/relaxation cycles could...
be repeated for at least 8 h, though over prolonged periods some stress relaxation in baseline force was noted. We could not determine a length dependence of force generation as classically done for striated muscle (9) because these preparations did not sustain stable forces when lengthened beyond the length formed in the mold. If untethered, the fibers shortened to approximately one-third the original formed length. Thus, at shorter lengths, the force would decrease. Serum induced dose-dependent contractions (Fig. 3), and a maximum isometric force of 106 ± 12 μN (n = 30) was developed in response to 30% serum. The times to half-maximal contraction and relaxation were 4.7 ± 0.3 and 3.1 ± 0.3 min (n = 22), respectively. Thrombin (2 U/ml) also elicited an increase in force, averaging 19.0 ± 2.0 μN (n = 13) above the prestimulus baseline.

As noted in previous studies (21), cytochalasin D (1 μM) reduced force in a serum-induced contracture to levels below the initial prestimulus levels (Fig. 4). The force remaining after cytochalasin D treatment was

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**Fig. 1.** Artificial fibers reconstituted from Swiss 3T3 fibroblasts. Fibroblast cells reaggregate to form a fiberlike preparation. Fibroblast cells (2 × 10^6 cells/ml) were cultured in collagen (0.5 mg/ml) medium matrix, as described in MATERIALS AND METHODS. Photograph shows a 100-mm petri dish in which 3 wells (0.8 × 4 cm × 0.5 cm deep) were cut in a layer of silicone rubber, previously cast in the dish. At 24-h intervals, an aliquot (2 ml) of the collagen/cell suspension was poured into 1 well and placed in a CO₂ incubator at 37°C. Photograph was taken at 48 h so that the total incubation time was 48 h for mold 1, 24 h for mold 2, and 30 min for mold 3.

**Fig. 2.** Serum-induced contraction of a reconstituted fibroblast fiber. A: After mounting in the isometric apparatus, fiber length was increased to its original length at formation in the mold (ST, left scale). After stress relaxation, the force scale was changed (right scale). Contraction was induced by 30% calf serum (CS). PSS indicates a return to the original physiological salt solution. Rapid spikes on solution changes are due to surface tension changes. B: a continuation of A. The serum-induced contraction was reversible, and the contraction-relaxation cycle was reproducible for at least 8 h.

**Fig. 3.** Contractile response of a reconstructed fibroblast fiber to CS. Mean dose-response curve for 10 fibers. Isometric force was normalized to the maximum value for each fiber. Maximum force was 101.8 ± 15.1 μN (n = 10). Fibers were incubated for short (30–60 min, ●; n = 5) or long terms (>15 h, ■; n = 10) in serum-free medium before force measurements. Inset: isometric force developed in response to cumulative addition of CS to a reconstituted fiber. PSS indicates a return to the original physiological salt solution.

**Fig. 4.** Cytoskeletal changes during a serum-induced contracture. A: Cytochalasin D (1 μM) caused a decrease in force. B: Barium chloride (25 mM) caused an increase in isometric force, and cytochalasin D treatment resulted in a decrease in force.
~20% of the total force in a maximum serum-induced contracture. Because cytochalasin D would disrupt actin-containing filament, forces due to both actin-myosin motors, analogous to “active force” or “tone” in smooth muscle, and cytoskeleton-related “passive” force, would be reduced. Thus an upper bound for the active force under unstimulated conditions would be 17.5% of the force developed on serum stimulation.

Our normal protocol was to place the fiber in serum-free medium the night before mechanical measurements. We investigated whether this serum-free period was required for the observed contractile responses. Test fibers were kept in serum until they were mounted for force measurements in serum-free media. After attainment of a steady baseline, which required between 30 and 60 min in serum-free conditions, isometric force was measured as a function of serum concentration. Figure 3 shows that the cumulative dose-response curves for fibers incubated for short (30–60 min) or long terms (>15 h) in serum-free conditions were identical. These experiments indicate that a prolonged serum-free period is not required by these fibers for contractile responses.

**Force-velocity relations.** These relations were studied by imposing a series of constant speed decreases in length and measurement of the consequent force responses. Figure 5A shows a typical experimental record for a single fiber composed of individual force responses to eight imposed shortenings of varying speeds. Force can be seen to continuously decline with the duration of shortening, similar to behavior reported for rat aorta (22) and hog coronary artery (17). Thus there is a family of force-velocity relations (Fig. 5B), each corresponding to the point in time at which the force values were measured. In the inset to Fig. 5B, \( V_{\text{max}} \) taken from each Hill equation was plotted as a function of the point in time at which the forces were measured. \( V_{\text{max}} \) decreases rapidly at first, then is relatively stable between 3 and 5 s. The velocities measured at early time points may in fact include discharge of series mechanical compliances, before a quasisteady state is achieved. In subsequent experiments, the force at 5 s was taken for all force-velocity relations. This point in time was chosen as it provided the widest range of measurable values over the differing experimental conditions. This provides a means for determining an operational, relative \( V_{\text{max}} \) for comparison of the different conditions.

Force-velocity relations were fitted with the Hill equation, \((F + a) \cdot (V + b) = b \cdot (F_0 + a)\), using a nonlinear least-squares routine (Origin). For maximum serum-induced contractures, \( F_0 \) was taken as the initial total force, and \( V_{\text{max}} \), the velocity at zero force, averaged 0.035 ± 0.002 Lo/s (n = 30); in the example shown in Fig. 5B, this was 0.030 Lo/s, where Lo was the initial length of the fiber. This velocity is in the range reported for tonic smooth muscles (24) and is considerably slower than striated muscle. \( a/F_0 \), a dimensionless curvature parameter, averaged 1.11 ± 0.07. This parameter, often inversely related to efficiency, is higher than generally observed for striated muscle (0.25) but is similar to that reported for the phasic rat portal vein (13). At the highest imposed shortening speeds, some compressive forces (negative forces) were measured. There does not appear to be any discontinuity in the force-velocity relations in this range. For unstimulated...
fibers \( (n = 30) \), these parameters averaged 0.015 \( \pm \) 0.001 \( \Delta L/\Delta L_0 \) and 1.96 \( \pm \) 0.20, respectively.

**Fiber stiffness.** Stiffness was measured by imposition of rapid (<1 ms) step changes in length. Typical responses are shown in Fig. 6A. After imposition of the step, the force response exhibited a peak value followed by stress relaxation. A plot of the peak force responses against the imposed step length change is shown in Fig. 6B. Typical of muscle behavior, the fibroblast peak force responses were linear in the region where stretches were imposed, and this linear range extended to short step decreases. In the serum-stimulated fibers, the slope of the linear relation between force and length (stiffness) was 23.4 \( \pm \) 0.92 mN/\( \Delta L_0 \) \( (n = 30) \). Extrapolation of the linear portion gives an intercept on the length axis of \(-0.0076 \pm 0.0006 \Delta L/\Delta L_0 \) \( (n = 30) \); i.e., a step of 0.76% \( \Delta L_0 \) is required to discharge the maximum isometric force. This value is lower than isolated smooth muscle cells (28) but comparable to that reported for skinned smooth muscle fibers (2).

**Effects of nocodazole and cytochalasin D on ultrastructure and mechanical parameters of reconstituted fibroblast fibers.** We used fluorescence microscopy to verify the ability of cytochalasin D and nocodazole to disrupt microfilaments and microtubules in these preparations, respectively. We selected concentrations based on the reported disruption of microtubules and actin filaments in cultured fibroblasts (21). As illustrated in Fig. 7A, intact microtubules were clearly visible in cells populating control fibers, whereas cells from fibers treated with 10 \( \mu \)M nocodazole for 10 min (Fig. 7B) exhibited a diffuse distribution of tubulin, indicating depolymerization of microtubules.

Organization of polymerized actin was assessed by staining cells with rhodamine-conjugated phalloidin. In control fibers (Fig. 8A), actin filaments were predominantly distributed at the cell periphery. Cells treated with CS (Fig. 8B) demonstrated increased staining for polymerized actin. Cytochalasin D treatment (Fig. 8C) disrupted actin morphology in CS-treated cells that resulted in clumping of polymerized actin.

With the use of the same concentrations of nocodazole and cytochalasin D, we assessed the effects of disruption of microtubules and actin filament networks on the mechanics of the fibroblast fiber. Addition of nocodazole to fibers precontracted with CS (30%) did not result in any statistical differences in the mean values, as summarized in Table 1. This average can be somewhat misleading in that there were appreciable

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**Fig. 6.** Stiffness measurements in reconstructed fibroblast fibers. A: time course of changes in length and force in nonstimulated fiber. B: the change in force plotted against the magnitude of the imposed length step (\( \Delta L/\Delta L_0 \)) in nonstimulated (○) and CS-stimulated (●) fibers. The peak force reached during rapid length steps (completed within 1 ms) is plotted against the amplitude of the imposed length steps. Regression lines (dashed lines) for force change vs. length change for stretches (positive \( \Delta L/\Delta L_0 \) values) are shown in the figure.
increases in force in 8 fibers and decreases in 13. Figure 9A shows the distribution of the responses of isometric force. Disruption of the microtubule network with nocodazole would be expected to significantly enhance the maximum shortening velocity if intact microtubules posed a significant internal resistance. The distribution of $V_{\text{max}}$ shows similar variability as isometric force (Fig. 9B). Nocodazole had no significant effect on the average $V_{\text{max}}$ (Table 1). As shown in Fig. 10, there is, however, a strong correlation between $V_{\text{max}}$ and force.

Fiber stiffness would also be expected to reflect any resistance attributable to microtubule networks. The distribution of stiffness values measured is given in Fig. 9C, and the dependence of stiffness on force is given in Fig. 11. Nocodazole did not alter the average stiffness (Table 1), but, like $V_{\text{max}}$, the level of force appeared to be the major determinant of stiffness. Thus the increase in force seen in some cases with nocodazole treatment was associated with an increase in resistance. This is the opposite of what was expected if reduction of microtubular mechanical resistance was responsible for the increase in force.

In contrast to nocodazole, cytochalasin D had major and consistent effects on the mechanical parameters studied (Fig. 10, Table 1). Isometric force was reduced to below the intrinsic levels of force in unstimulated fibroblasts. Velocity was proportionately reduced (Fig. 10). Stiffness was decreased to $\sim50\%$ of the initial unstimulated values; however, the precision of these measurements was reduced due to the low force remaining after cytochalasin D treatment (Fig. 11).

Effects of nocodazole and cytochalasin D on $[\text{Ca}^{2+}]_i$. To further delineate the mechanisms underlying the effects of microtubule or microfilament disruption on contractility, we measured $[\text{Ca}^{2+}]_i$ using ratiometric fluorescence spectroscopy and the $\text{Ca}^{2+}$-sensitive dye fura 2. Nocodazole (10 $\mu$M) elicited a small increase in force with little effect on $[\text{Ca}^{2+}]_i$ (Fig. 12). Addition of serum produced a transient increase in $[\text{Ca}^{2+}]_i$ that was smaller ($72.1\% \pm 3.8$, $n = 8$, $P = 0.002$) than that seen in the absence of nocodazole. The addition of serum in the presence of nocodazole elicited a force response that was nearly identical ($97.4\% \pm 0.9$, $n = 8$, $P = 0.02$) to that in its absence.

Cytochalasin D (1 $\mu$M) also had little effect on baseline $[\text{Ca}^{2+}]_i$ and reduced ($89.2\% \pm 1.9$, $n = 7$, $P < 0.001$), but did not eliminate, the increase in $[\text{Ca}^{2+}]_i$ in response to serum (Fig. 13). In contrast to nocodazole treatment, cytochalasin D treatment nearly abolished ($15.8\% \pm 0.8$, $n = 7$, $P < 0.001$) the increase in force in response to addition of serum.

**DISCUSSION**

We have shown that artificial fibers composed of fibroblasts grown in a culture media including collagen provide a unique model for measurement of mechanical parameters in nonmuscle cells. Because cytochalasin D nearly abolishes mechanical responses, these mechanical parameters reflect aggregate cellular properties rather than those of the collagen matrix in which the cells are grown. In the absence of cells, collagen alone does not form fibers or support mechanical loads.

The aggregated cells generate force under unstimulated conditions. This is demonstrated by the ability of the fiber to shorten and by the inhibition of force by cytochalasin D. Both serum and thrombin elicit dose-dependent increases in isometric force. The response to serum of these Swiss fibroblasts was approximately fivefold greater than thrombin. The maximum isometric forces measured in this study were on the order of 150 $\mu$N. If one assumes that the original $4 \times 10^6$ fibroblasts were evenly distributed throughout the reconstituted fiber, and were 100 $\mu$m long with a 10-$\mu$m diameter, then the cellular cross-sectional area of the fiber can be calculated as $\sim0.8$ mm$^2$. The actual fiber cross-sectional area is difficult to determine exactly but
### Table 1. Mechanical properties of fibroblast fibers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Serum</th>
<th>Serum + Nocodazole</th>
<th>Cytochalasin D</th>
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<tbody>
<tr>
<td>Total isometric force, mN</td>
<td>0.053 ± 0.003</td>
<td>0.159 ± 0.010*</td>
<td>0.130 ± 0.014*</td>
<td>0.035 ± 0.008*‡</td>
</tr>
<tr>
<td>Velocity, V_{max}, L/s</td>
<td>0.015 ± 0.001</td>
<td>0.035 ± 0.002*</td>
<td>0.027 ± 0.004†</td>
<td>0.008 ± 0.002‡</td>
</tr>
<tr>
<td>a/P_o</td>
<td>1.960 ± 0.200</td>
<td>1.110 ± 0.070†</td>
<td>1.520 ± 0.220</td>
<td>3.670 ± 0.730†‡</td>
</tr>
<tr>
<td>b</td>
<td>0.022 ± 0.003</td>
<td>0.035 ± 0.003*</td>
<td>0.031 ± 0.004†</td>
<td>0.019 ± 0.002‡</td>
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<tr>
<td>Stiffness, mN/L_o</td>
<td>17.960 ± 0.850</td>
<td>23.430 ± 0.920†</td>
<td>21.610 ± 1.180†</td>
<td>12.370 ± 1.070‡</td>
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<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>21</td>
<td>15</td>
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Values are means ± SE. Maximum velocity (V_{max}), a/P_o, and b were calculated from Hill equation fitted to force-velocity curve, where P_o is isometric force. Stiffness was determined as the slope of the linear relation between force and length, as shown in Fig. 6. Calf serum (30%), nocodazole (10 µM), and cytochalasin D (1 µM) were used. Significantly different from the value in nonstimulated fibers were †P < 0.05 and *P < 0.01, and from the value in serum-stimulated fibers, ‡P < 0.01 (ANOVA). A subset of the data on control and serum-treated fibers was reported by Obara et al. (23), 1995.

Fig. 8. Immunofluorescent micrographs of fibroblast fibers labeled with rhodamine. Polymerized actin was visualized with rhodamine-conjugated phalloidin control fibers (A), fibers treated with CS (B), and fibers treated with CS followed by cytochalasin D (C; 1 µM). Scale bar (A) applies to all panels. Actin polymerization was enhanced by CS but disrupted by cytochalasin D.
is roughly between 1 and 4 mm². Thus this estimate is not unrealistic and is comparable to some vascular smooth muscle tissue in which smooth muscle cells account for 25% of the vessel wall. Thus force per cellular cross-sectional area would be 0.2 mN/mm². This is considerably less than that for smooth muscle, in which tissue values range from 10 to 200 mN/mm², and potentially larger when translated to smooth muscle cellular cross-sectional areas. However, our calculated value of 0.2 mN/mm² is of similar magnitude to those estimated for fibroblasts from wound contraction models in skin preparations (15).

Hyperbolic force-velocity relations, similar to tonic smooth muscle, were observed in these reconstituted fibers. As force continuously declined with time during the imposed constant shortening, assignment of a maximum velocity depended on the time of measurement. This is similar to that observed in studies of smooth muscle, in which, for example, unloaded velocities showed a similar slowing with time. Whether this is due to some internal resistance, as suggested for smooth muscle (12), or a possible dependence on length

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**Fig. 9.** Distribution of individual values for force (A), $V_{\text{max}}$ (B), and stiffness (C) under control conditions and in response to serum, nocodazole (serum + Noco), and CytoD. The responses for each individual fiber are designated by the same symbol and connected by dotted lines.

**Fig. 10.** Effects of CS, nocodazole, and cytochalasin D on the relation between maximum shortening velocity and force. Force and $V_{\text{max}}$ were normalized to those in the resting state. Nocodazole and cytochalasin D were applied after the serum-induced contraction reached a plateau. ■, CS (Ser, 30%); ●, nocodazole (Noco, 10 μM); ▲, cytochalasin D (CytoD, 1 μM). Regression lines for CS (dashed line), nocodazole (broken line), and cytochalasin D (solid line) are shown. The level of isometric force, independent of how it is achieved, appears to be the major determinant of $V_{\text{max}}$.

**Fig. 11.** Effects of CS, nocodazole, and cytochalasin D on the relation between stiffness and force. Force and stiffness were normalized to those in the resting state. Nocodazole or cytochalasin D were applied after the serum-induced contraction reached a plateau. Symbols and regression lines have the same meanings as in Fig. 10. Note that nocodazole has little effect on the relation between stiffness and force, whereas cytochalasin D elicits major changes.
(11), is not known. However, the maximum velocities deduced from these force-velocity relations were similar to those visually measured in unloaded chicken embryo fibroblasts cells on cutting from the naturally occurring constraints in the forming molds (21). These maximum velocities are much slower than striated muscle but similar to those reported for the slower, tonic smooth muscles, such as the aorta (25). Velocity and myosin ATPase activities are generally correlated. The low velocity of these fibroblast fibers is consistent with the low-myosin ATPase rates reported for non-muscle myosin. The actin-activated Mg$^{2+}$-ATPase reported for clonal mouse fibroblast myosin is 90 nmol·min$^{-1}$·mg$^{-1}$ (1). This can be compared with 89 nmol·min$^{-1}$·mg$^{-1}$ for platelet myosin and 51 nmol·min$^{-1}$·mg$^{-1}$ reported for gizzard smooth muscle measured under similar conditions (26).

In assessment of the data as a whole, the level of isometric force appeared to be the major determinant of the speed of shortening. In Fig. 10, the maximum velocities under the various conditions studied were plotted against the level of isometric force. Whether the variations in force were due to the natural variations in response to serum or modified by nocodazole or cytochalasin D, higher levels of force were strongly correlated with higher velocities.

Fibroblast fiber stiffness was also assessed in this model and was found to be similar to that reported for smooth muscle cells but considerably higher than that for isolated smooth muscle tissue. This may reflect the differences in structures through which force is transmitted. Stiffness was related to the level of isometric force (Fig. 11), though this dependence was not as highly correlated as $V_{\text{max}}$ and was sharply reduced after cytochalasin D treatment. The relations between stiffness and force were similar for serum contractures in the presence or absence of nocodazole, which suggest that microtubules are not a major determinant of this parameter. The linear dependence of stiffness on force of these cell aggregates is similar to that observed for individual cells and predicted by tensegrity models (27).

We tested whether microtubules constituted significant mechanical resistance or internal load by measuring the effects of nocodazole, an agent known to disrupt microtubules (16), on shortening velocity and stiffness. Our immunomicroscopy confirmed that nocodazole at 10 μM also disrupted microtubule structures in fibro-
blasts in our reconstituted fibers (Fig. 7). Nocodazole has been shown in previous studies to increase isometric force in stimulated fibroblast fibers (21) that would be consistent with a reduction in internal resistance opposing the force generated by actin-myosin motors. In contrast to previous studies, we did not consistently observe an increase in force in response to nocodazole (Fig. 9A). However, in our studies, nocodazole was added to cells previously contracted with CS. Contraction with CS sharply decreased the additional response to nocodazole in chicken embryo fibroblasts (18). This finding suggests that, in contrast to the predictions of the tensegrity model, nocodazole and CS may cause contraction through a common signaling pathway that may become saturated by CS, thereby diminishing the nocodazole response.

\[ V_{\text{max}} \] would be expected to be very sensitive to such an internal resistance. Based on the measured force-velocity relations (Fig. 5B), eliminating an internal load with a magnitude of only 20% of the maximum isometric force would increase fiber \[ V_{\text{max}} \] by 40%. The effects of nocodazole on \[ V_{\text{max}} \] depended on its effects on force, which were variable. However, there was no net effect of nocodazole on \[ V_{\text{max}} \] of serum-stimulated fibers (Table 1).

The relation between the level of isometric force and \[ V_{\text{max}} \] seen after nocodazole treatment was also similar to that observed for serum stimulation alone (Fig. 10). This provides evidence against the presence of a specific mechanical resistance that can be attributed to microtubules. This is further supported by the effects of nocodazole on fiber stiffness. When nocodazole treatment was associated with increased force, stiffness increased. This is the opposite to expectation if disruption of microtubules removed an internal resistance. The increase may be attributed to more actin-myosin cross bridges under these conditions. This is consistent with recent studies suggesting that nocodazole treatment increases myosin light chain phosphorylation in fibroblasts (18).

The effects of nocodazole on both stiffness and \[ V_{\text{max}} \] appear to be mediated through its effects on force. Larger forces, independent of how they were generated, were associated with greater \[ V_{\text{max}} \]. This would be consistent with some type of nonmicrotubule internal resistance in the aggregated cell fiber. If such a resistance was constant, higher forces would lead to relatively less loading and faster velocities. This would also be consistent with the decrease in shortening velocity with time, as has been proposed for smooth muscle cells (12).
In contrast to nocodazole, cytochalasin D, which disrupts actin filaments (3), had dramatic inhibitory effects on the mechanical process(es) underlying all measured parameters. The effects of cytochalasin D on these mechanical parameters was paralleled by its depolymerization of actin filaments in the fibroblast fiber (Fig. 8). Thus the ability to develop force and to shorten is highly dependent on the integrity of the actin filament network. The decreases in both stiffness and V_{max} by cytochalasin D were parallel to that of its inhibition of force and consistent with force being the primary determinant of these parameters. This would be consistent with actin-myosin interaction being the primary locus of force generation in these fibers.

It is also of interest to consider the possibility of linkage between actin microfilaments and microtubules, as suggested by actin binding properties of some microtubule-associated proteins (8, 14). If, by some manner, actin and microtubule networks are linked, and nocodazole destroys the linkage decreasing parallel cross bridges, then force, as well as stiffness, would be expected to decrease. This is what is observed. If these cross bridges are “lost” to force generation, then this may underlie the loss of force with nocodazole observed in some cases. On the other hand, if the loss of parallel cross bridges is translated into more series cross bridges, then one might anticipate V_{max} to increase, which was not observed. Further speculation in lieu of high-resolution ultrastructure studies is not warranted. However, these mechanical data provide the first insight into the relations between force, stiffness, and shortening velocity in a nonmuscle contractile system.

Because cytochalasin D and nocodazole appear to be mediated via their effect on [Ca^{2+}], the mechanism of activation of nonmuscle myosin is controversial (20, 23), but [Ca^{2+}] is likely a key second messenger. Treatment with nocodazole reduced the increase in [Ca^{2+}] in response to serum by ~30%, but had little effect on the developed force. Cytochalasin D, which dramatically reduced force, had an even lesser effect than nocodazole on the Ca^{2+} response to serum. Thus it is unlikely that the effects of either were mediated primarily through effects on [Ca^{2+}].

In summary, reconstituted fibers formed by fibroblasts cultured in a collagen gel can be used for precise mechanical measurements in aggregated nonmuscle cells. Although the isometric force generated by these reconstituted fibers is significantly lower, maximum velocity and compliance are similar to that of smooth muscle fibers. In terms of the contractile response to serum, actin microfilaments play a major role in the mechanical properties of the reconstituted fibers, as might be expected for a myosin motor-driven system. Microtubules do not make a major contribution to an internal mechanical resistance or load against which the myosin system has been proposed to operate (7). Because the composition of the major components in these cultured cells can be readily manipulated by genetic techniques (23), this model system may provide a unique approach to the understanding of contractile mechanisms in nonmuscle as well as cultured muscle cells.

REFERENCES


