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Review

Global cytoskeletal control of mechanotransduction in kidney epithelial cells

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Abstract

Studies of mechanotransduction mediated by stress-sensitive ion channels generally focus on the site of force application to the cell. Here we show that global, cell-wide changes in cytoskeletal structure and mechanics can regulate mechanotransduction previously shown to be triggered by activation of the mechanosensitive calcium channel, polycystin-2, in the apical primary cilium of renal epithelial cells [S.M. Nauli, F.J. Alenghat, Y. Luo, E. Williams, P. Vassilev, X. Li, A.E. Elia, W. Lu, E.M. Brown, S.J. Quinn, D.E. Ingber, J. Zhou, Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. Nat. Genet. 33 (2003) 129–137]. Disrupting cytoplasmic microfilaments or microtubules in these cells eliminated fluid shear stress-induced increase of intracellular calcium. Altering the cytoskeletal force balance by inhibiting actomyosin-based tension generation (using 2,3-butanedione monoxime), interfering with microtubule polymerization (using nocodazole, cochicine, or taxol), or disrupting basal integrin-dependent extracellular matrix adhesions (using soluble GRGDSP peptide or anti-β1 integrin antibody), also inhibited the calcium spike in response to fluid stress. These data indicate that although fluid stress-induced displacement of the primary cilium may be transduced into a calcium spike through activation of polycystin-2 and associated calcium-induced calcium release from intracellular stores, this mechanotransduction response is governed by global mechanical cues, including isometric tension (prestress) within the entire cytoskeleton and intact adhesions to extracellular matrix.

Keywords: Mechanotransduction; Calcium; Cytoskeleton; Extracellular matrix; Cilia; Integrin

Introduction

Mechanotransduction—the process by which cells sense mechanical force and convert it into a biochemical response—impacts tissue development as well as many diseases by altering cell physiology, including growth, differentiation, migration, gene expression, protein synthesis, and apoptosis [1]. Recent work on the molecular mechanism of mechanotransduction has revealed that specific cell surface molecules, including stress-sensitive ion channels and integrin receptors, play a key role in this response [2]. Ion channels can alter transmembrane ion transport in response to mechanical perturbations, including application of fluid shear stress [3,4]. Integrins are adhesion receptors that anchor cells to extracellular matrix and provide a preferential path for mechanical force transfer across the plasma membrane [5]. These forces impact various cytoskeletal-associated signaling molecules within the focal adhesion complex on the internal face of the adhesion site (review in Ref. [2]), and control the dynamics of focal adhesion assembly [6,7].

Most studies of mechanotransduction focus on how forces applied to the cell surface influence chemical signaling activities near the site of force application, such as ion flux across the apical membrane in response to fluid shear or focal adhesion remodeling following matrix

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distortion [8–10]. In the case of integrin-mediated mechanical signaling, the physiological impact of these local mechano-sensitive signaling events appear to be governed by global changes in cell shape [11]. For example, mechanical forces applied to integrins produce different effects on focal adhesion assembly in round vs. spread cells [12,13]. The degree to which a cell physically distorts when bound to extracellular matrix (through integrins) also can feedback to control cell growth, contractility, differentiation, motility, and apoptosis [14–16]. Changes in cell shape influence many of these behaviors as a result of alterations in cytoskeletal structure or variations in the level of isometric tension (prestress) within the cytoskeleton [17,18]. In the present study, we set out to explore whether altering cytoskeletal structure or mechanics also can influence mechanically induced ion signaling in an experimental system where the cell surface molecules that initiate the cellular mechanotransduction response have been identified.

Murine embryonic kidney epithelial (MEK) cells respond to increases in fluid shear stress across their apical surface by rapidly increasing calcium influx through stress-sensitive ion channels, and then further amplifying the response through calcium-induced release of calcium from intracellular stores [4]. This response is initiated by flow-induced bending of the primary cilium on the apical surface of these cells [4,19]. Primary cilia are also required for induction of intracellular calcium spikes in response to physiological levels of fluid shear stress in canine kidney epithelial (MDCK) cells [19], and a similar cilium-dependent mechanism mediates establishment of left–right body asymmetry by nodal cells during embryogenesis [20].

The stress-induced calcium response in kidney epithelial cells is mediated by polycystin-1 and polycystin-2 proteins that preferentially localize to the base of the primary cilium [4,20,21]. Flow-induced bending of the cilium apparently results in conformational changes of polycystin-1 that transduce the mechanical signal into a chemical response via activation of associated polycystin-2, which functions as a calcium channel [4,22–24]. Once calcium crosses the plasma membrane via this mechanically triggered pathway, it induces release of additional calcium from intracellular stores via a ryanodine-sensitive mechanism that further amplifies the response [4].

Despite the clear requirement for polycystins in mediating the calcium response to fluid flow sensation in renal epithelial cells, it is important to consider the mechanical prerequisites for mechanosensation in these cells. Primary cilia that protrude from the apical surface of the cell into the tubular lumen contain ordered microtubules in a 9 + 0 array that are composed of acetylated α-tubulin and detyrosinated tubulin [25,26]. Though the cilium attaches to a small (<1 μm) region on the apical surface, it connects with the cortical (submembranous) actin cytoskeleton through its basal body just inside the apical cell surface [27]. This cortical network, in turn, links to the remainder of the deeper cytoskeleton in the underlying cytoplasm (composed of microfilaments, microtubules, and intermediate filaments), and from there to cell–matrix and cell–cell adhesions at the cell periphery [28]. Cytoskeletal microtubules work together with integrins in extracellular matrix adhesions and cadherins in cell–cell junctions to resist the pull of tensile forces generated within the actin cytoskeleton, and thereby establish a mechanical force balance that stabilizes the shape of the entire cell [18,28,29].

This balance of compression and tension on a global scale could potentially influence how a cell carries out mechanotransduction through stress-sensitive ion channels within a local microdomain on the cell surface. For example, in integrin-based mechanotransductory systems, cells respond differently to mechanical stimuli when the baseline cytoskeletal tension is altered [7,9,30]. However, whether this effect is mediated through global cytoskeletal control of the initial mechanotransduction events, or through modulation of later signaling events, remains unclear. Thus, in the present study, we used the mechanosensitive calcium signaling system in MEK cells to explore whether the mechanotransduction events triggered by fluid shear are indeed sensitive to changes in the structure and mechanics of the whole cell.

Methods

Embryonic kidney cell culture

Wild-type murine kidney epithelial (MEK) cells cultured from the collecting ducts of embryonic mice were grown in DMEM media containing 2% fetal bovine serum, 0.75 μg/l interferon-γ, 1.0 g/l insulin, 0.67 mg/l sodium selenite, 0.55 g/l transferrin, 0.2 g/l ethanolamine, 36 ng/ml hydrocortisone, 0.10 μM 2,3,5-Triido-L-thyronine, 100 units of penicillin-G (base) in combination with 0.30 mg/ml additional glutamine, 100 μg of streptomycin sulfate, and 0.1 mM citrate to maintain penicillin potency, as previously described [4]. All cell culture supplements were obtained from Invitrogen, CA, except for interferon-γ, hydrocortisone, and 2,3,5-Triido-L-thyronine, which were obtained from Sigma. Sodium pyruvate (11.0 g/l) was added to maintain cell viability in suspension media. In this study, only epithelial cells from the 3rd to 18th passages after sorting were used. Immunofluorescence staining technique as described previously was used with mouse acetylated α-tubulin (Sigma), rabbit anti-β1 integrin (M106, Santa Cruz).

Shear flow system

A parallel-plate flow chamber (Glycotech) was used to subject adherent cells to shear stress on the microscope with phase or fluorescent imaging. The region of flow

exposure was 2.5-cm long and 1.0-cm wide with a clearance height of 0.025 cm. Shear stress was controlled by adjusting the volume flow rate, $\tau_w = 6\mu Q/a^2 b$, where τ_w is the shear stress (dyn/cm^2) at the cell surface, μ is the viscosity of the media (0.0076 poise), Q is the volume flow rate (ml/s), a is the clearance height (0.025 cm), and b is the flow area width (1.0 cm). For all flow experiments, a nonfluorescent, CO_2-independent medium (pH 7.3) was used that contained (in mM): $CaCl_2$ (1.26), $MgSO_4$ (0.81), KCl (5.36), KH_2PO_4 (0.44), NaCl (137), Na_2HPO_4 (0.34), D-glucose (5.55), L-glutamine (2.0), sodium pyruvate (1.0), HEPES (20.0), 1% bovine serum albumin, and MEM essential and nonessential amino acids (Sigma).

Magnetic twisting cytometry

We used magnetic twisting cytometry [5,31] to determine whether direct application of mechanical stress to integrins is sufficient to activate calcium signaling in MEK cells. Carboxylated ferromagnetic beads (4.5-μm diameter; Spherotech) were coated with RGD (Peptite-2000, 0.25 mg/ml, Integra Life Sciences) in 0.1 M sodium phosphate buffer (pH 6.2) with 1.25 mg/ml *N*-(3-Dimethylaminopropyl)-*N*′ethylcarbodiimide hydrochloride (EDAC, Sigma) for 2 h at 22°C. Beads were centrifuged and washed three times with PBS and stored at 4°C before use. RGD-coated beads (approximately 20 beads/cell) were incubated with cells for 30 min, washed with phosphate-buffered saline with calcium (PBS), and placed in experimental medium (see above). To apply a torque to cell-bound beads, they were exposed to a brief (10 μs) but strong (1000 Gauss) horizontal magnetic field to align the magnetic dipoles of the beads. They were then exposed to a prolonged (10 min) but weaker (30 Gauss) vertical field that twists the beads, as previously described [5,31].

Ca^{2+} imaging

MEK cells were grown for at least 2 days in the absence of interferon-γ to induce optimal differentiation. The nonfluorescent, CO_2-independent media described above was used for Fura-2 Ca^{2+} imaging during flow. In some experiments, cells were incubated with cytochalasin B or D (10 μM), latrunculin B (2 μg/ml), colchicine (10 μM), Y27632 (10 μM), 2,3-butanedione monoxime (BDM; 10 mM), nocodazole (10 μg/ml), or taxol (paclitaxel; 100 μM) for 30 min before the flow trial. We have previously confirmed that these treatments specifically disrupt or modulate their respective cytoskeletal targets in various cell types [17,32,33]. In other experiments, cells were incubated with soluble activating (BD15) or nonactivating (K20) anti-β1 integrin antibodies at 10 μg/ml for 30 min, with RGD-coated microbeads (approximately 20 beads/cell) for 30 min, or with the soluble cell-binding peptide from fibronectin, GRGDSP (American Peptide), or a control peptide (GRGESP, American Peptide) at 15 μM for 10 min. Regardless of treatment, all cells were incubated for 30 min with the Ca^{2+}-sensitive probe Fura2-AM (5 μM) at 37°C, washed three times with PBS, and placed in the parallel plate flow chamber. The chamber was positioned under a Nikon Diaphot microscope equipped with a CCD camera using IPLab software for Macintosh. Paired Fura images at excitation wavelengths of 340 and 380 nm were captured every 5 s. After equilibration in the microscopy media for 10 min, the cells were exposed to a fluid shear stress of 0.75 dyn/cm^2. The same equilibration and image timings were employed for the magnetic twisting studies.

Data analysis and statistics

The Ca^{2+} level relative to baseline was ratiometrically calculated using R_{min} and R_{max} values of 0.3 and 6.0, respectively, where the cytosolic free Ca^{2+} concentration is proportional to $(R - R_{min})/(R_{max} - R)$. R is the ratio of 510-nm emission intensity when excited at 340 nm to 510-nm emission intensity when excited at 380 nm; R_{min} and R_{max} are ratios at zero and saturating (10 mM) Ca^{2+}, respectively, as determined previously [4]. All values represent mean ± standard error for 50 cells per experimental condition, and each experiment was repeated between two and five times with similar results. Comparisons between means were performed using paired Student's t test, and statistical significance implies $P < 0.05$.

Results

When MEK cells were loaded with the Ca^{2+}-binding dye, Fura-2, exposed to the abrupt onset of physiologically relevant levels of fluid shear (0.75 dyn/cm^2) and analyzed by microfluorimetric ratio imaging, a rapid rise in intracellular Ca^{2+} could be detected throughout the cell population, peaking roughly 10–15 s after flow stimulation (Fig. 1). Ca^{2+} levels then quickly decreased, returning to baseline approximately 20–30 s later. We recently showed that this response is triggered by polycystin-1 and polycystin-2 at the base of the primary cilium on the apical cell surface, and associated calcium-induced calcium release from intracellular stores in nearly identical experiments with the same cell type [4]. To explore whether this stress-dependent change in calcium signaling is sensitive to global changes in cell architecture, we first chemically manipulated the structure and mechanics of cytoskeletal elements that are not present within the primary cilium. Disrupting actin microfilaments by pretreating the cells with 10 μM cytochalasin B (Fig. 1a), 10 μM cytochalasin D (not shown), or 2 μg/ml latrunculin B (Fig. 1b) all blocked the stress-induced Ca^{2+} response. Moreover, the calcium spike was similarly inhibited using drugs that inhibit actomyosin-based tension generation without compromising cytoskeletal integrity, including the myosin ATPase inhibitor, BDM (10

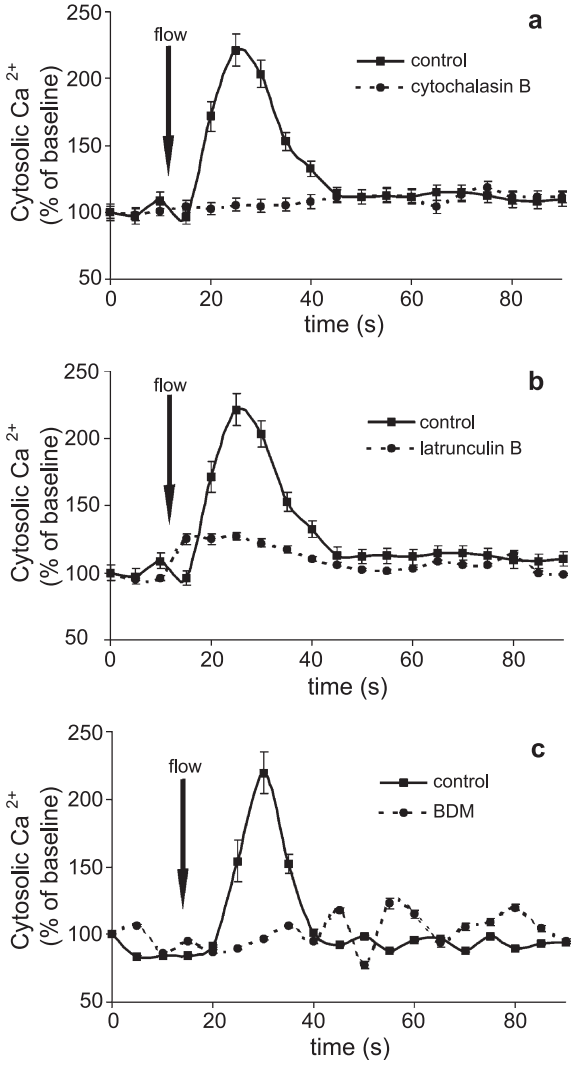

Fig. 1. Disruption of cytoplasmic microfilaments and inhibition of actomyosin-based tension generation eliminates the flow-induced Ca^{2+} response in MEK cells. Confluent and fully differentiated MEK cells preloaded with Fura-2 dye were subjected to fluid flow with shear stress at the apical cell surface of 0.75 dyn/cm^2 in the presence or absence of various cytoskeletal modulators, and subjected to ratio imaging to determine intracellular Ca^{2+} concentrations. Under control conditions, intracellular Ca^{2+} increased to over twice the baseline within approximately 10–15 s after onset of flow (large arrow), and returned to baseline within 30 s later. Treatment of cells with cytochalasin B (a) or latrunculin B (b) to disrupt actin stress fibers eliminated the Ca^{2+} response to flow, as did exposure to BDM (c) to inhibit cytoskeletal tension generation.

mM, Fig. 1c), and the rho-associated kinase (ROCK) inhibitor, Y27632 (10 nM, not shown).

Another way to alter cytoskeletal prestress (isometric tension) in the cell is to change the state of polymerization of microtubules that, in part, serve to resist the pull of surrounding contractile microfilaments [18,28]. Pretreating the MEK cells with either 10 μM colchicine (Fig. 2a) or 10 μg/ml nocodazole (Fig. 2b), which depolymerize microtubules, completely blocked the normal calcium response to flow. Furthermore, addition of 100 μM taxol, which induces polymerization of microtubules and mechanically stabilizes them [5,34], also suppressed the stress-induced calcium signaling response (Fig. 2c). Therefore, both the absence and overrigidity of the microtubule network prohibit cilia-mediated mechanosensation.

Cytoskeleton-modulating drugs alone, however, cannot give a complete picture of the role of whole-cell mechanics in flow sensation, because cilia are composed of microtubules and may be associated with actin at their base [26,27]. Another way to alter the global shape of the cytoskeleton, and to change cytoskeletal prestress, is to dislodge the cell's basal anchors to the underlying extracellular matrix that normally balance cytoskeletal-based traction forces [28]. Past studies have shown that the cell's basal adhesions to extracellular matrix can be dislodged by adding soluble GRGDSP peptide that contains the cell-binding sequence of fibronectin [35]. When we added 15 μM GRGDSP to MEK cells, the Ca^{2+}

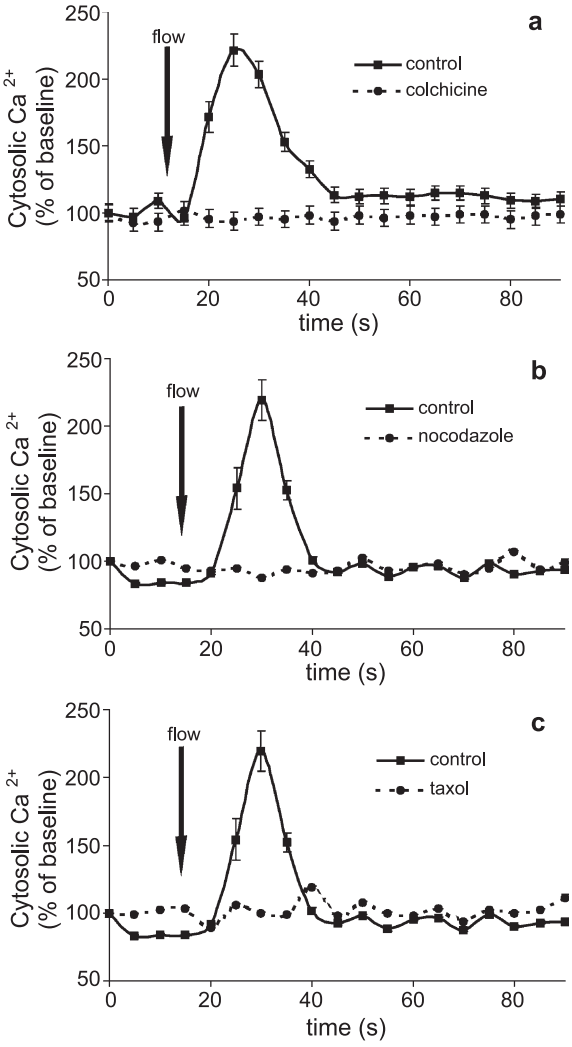

Fig. 2. Disrupting or stabilizing microtubules eliminates the flow-induced calcium response. Pretreatment of MEK cells with colchicine (a) or nocodazole (b) to disrupt microtubules blocked the cell's calcium signaling response to physiological levels of fluid shear stress. Stabilization of microtubules with 100 μM taxol (c) also abated the flow response.

flow response was completely eradicated within 10 min (Fig. 3a) before any significant change in cell morphology could be observed, although cells fully detached from the dishes at later times (>30 min) in response to flow. In contrast, treatment with the same concentration of a control hexapeptide (GRGESP) that does not compete for integrin binding or dislodge cells did not significantly inhibit stress-induced calcium signaling (Fig. 3a). Additionally, pretreatment of cells with a soluble, activating anti-β1 integrin antibody (BD15) completely blocked the response to flow as well, whereas a nonactivating antibody against the same subunit (K20; [36]) had no effect (Fig. 3b).

One possibility is that this inhibitory effect on calcium signaling could result from binding of these ligands to apical integrins, perhaps associated with the primary cilium. To explore this possibility, we used magnetic twisting cytometry to apply a controlled torque (shear stress) directly to apical integrins via surface receptor-bound magnetic beads

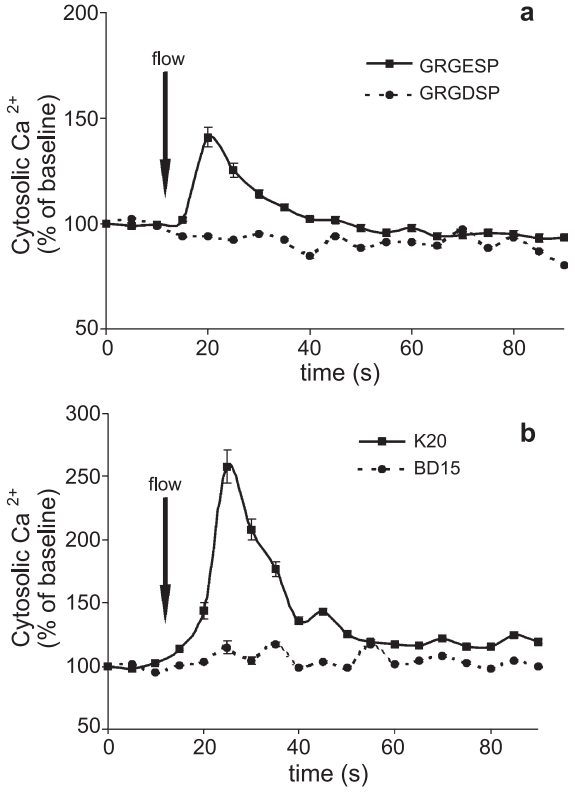

Fig. 3. The flow-induced Ca^{2+} response requires stable integrin-dependent adhesions at the cell base. (a) Treatment of MEK cells for 10 min with soluble GRGDSP (15 μM), which competes with immobilized ECM proteins for integrin binding, resulted in loss of their sensitivity to fluid flow and suppression of calcium signaling prior to inducing cell retraction or detachment. In contrast, similar treatment with a control soluble peptide—GRGESP—failed to eliminate the calcium signaling response. (b) Treatment of cells with soluble BD15 antibody that specifically competes with immobilized integrin ligands for binding to β1 integrins also suppressed the flow-induced calcium signaling response, whereas K20 antibody, which binds β1 integrin without interfering with ligand binding, did not affect flow sensation.

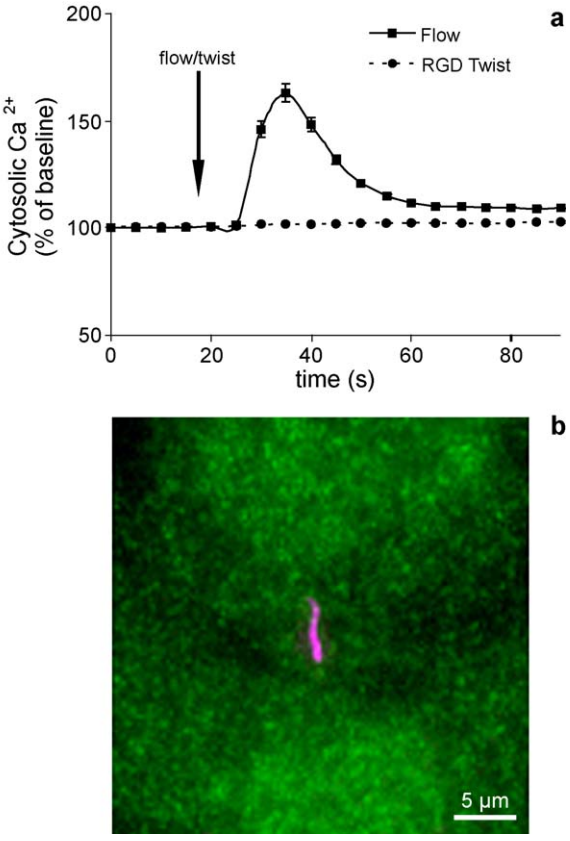

Fig. 4. Force application to apical β1 integrins, which do not colocalize with primary cilia, failed to produce a calcium signaling response. (a) When cells bound to RGD beads were subjected to a level of shear stress similar to that exerted by fluid shear (0.75 dyn/cm^2) using magnetic twisting cytometry, no calcium signaling was observed. (b) When these fully differentiated MEK cells were double-stained for acetylated α-tubulin (violet) to visualize the primary cilium and β1 integrins (green) on the apical cell surface using confocal microscopy, there was no detectable colocalization.

that were precoated with a RGD-containing synthetic peptide that ligates β1 integrin [5,31]. Application of a level of physiological shear stress similar to that applied in the fluid flow studies (0.75 dyn/cm^2) to apical integrins on MEK cells failed to induce a Ca^{2+} response (Fig. 4a). There also was no response when we applied higher levels (15–30 dyn/cm^2) of twisting or pulling (tensional) forces on similar beads using a magnetic microneedle [37] (not shown). Furthermore, no colocalization of integrins with the primary cilium could be detected when we double-stained MEK cells for acetylated α-tubulin and β1 integrin and analyzed the pattern using confocal microscopy (Fig. 4b); acetylated α-tubulin was tightly limited to the well-defined cilia, whereas β1 integrins distributed diffusely over the remainder of the apical cell membrane.

Discussion

These studies were carried out to explore whether cellular mechanotransduction events that are triggered at

the plasma membrane through a well-defined molecular mechanism involving stress-sensitive ion channels can be modulated by global changes in cell architecture. To approach this question, we studied renal epithelial (MEK) cells that sense apical fluid shear stress through bending of the primary cilium and activation of associated polycystin-2 calcium channels; this initiating event is then amplified through calcium-induced release of additional calcium from intracellular stores [4].

Our results show that this polycystin-2-based mechanotransduction mechanism is governed by the overall structural status of the entire cytoskeleton. Treating cells with various chemical agents that disrupt microfilaments (cytochalasins B & D, latrunculin B) or microtubules (colchicine, nocodazole), or that stabilize microtubules (taxol), eliminated the flow-induced intracellular calcium response. The response was similarly inhibited when cytoskeletal tension generation (and hence cytoskeletal prestress) was dissipated without compromising cytoskeletal integrity using BDM and Y27632 that inhibit myosin ATPase and ROCK, respectively [38,39]. Dissipation of cell tension by disrupting basal focal adhesions using synthetic GRGDSP peptide or specific anti-β1 integrin antibody also completely inhibited the mechanotransduction response, whereas control probes were ineffective. These findings support the hypothesis that mechanotransduction events that are triggered at the surface membrane can be modulated by the global mechanical state of the entire cell, including the level of prestress in the cytoskeleton [6,11,40].

Because the cilium is composed of microtubules, part of the effects of the microtubule-disrupting agents may be due to disruption of primary cilium architecture or mechanics. However, the finding that a variety of drugs that work through different mechanisms (e.g., integrin disruption) and other cytoskeletal filament systems (e.g., microfilaments, actomyosin-based tension generation) produced similar inhibitory effects indicates that mechanical signaling triggered by polycystin-2 activation at the cell apex is sensitive to the architecture of the entire cell and to internal mechanical forces that are transmitted through the cytoskeleton from the basal to the apical surface [6,18,41,42]. Because microtubules and extracellular matrix adhesions generate prestress in the cell by resisting the pull of the contractile microfilament cytoskeleton, any alteration in internal cell tension alters cytoskeletal mechanics as a whole [18,28]. Changes in the integrity of microtubules also can alter myosin light chain phosphorylation and thereby further alter the level of isometric tension in the cytoskeleton [18,43].

While this is the first investigation of the cytoskeleton in the response of kidney epithelium to fluid flow, microtubule disruption has been previously shown to inhibit shear-induced vasodilation of arterioles and migration [44,45] and flow-induced suppression of endothelin-1 transcription in endothelium [46–48]. Although these endothelial responses may be due to interference with events, such as cell migration and polarity [49,50], the present findings raise the possibility that microtubule disruption also might influence the initial mechanotransduction events at the surface membrane, although those cells do not commonly exhibit primary cilia in culture. Thus, our findings suggest that the role of microtubules in mechanotransduction may extend beyond their function in the primary cilium.

If whole-cell cytoskeletal force transmission is a prerequisite for the lumenal flow-induced Ca^{2+} response in kidney, the logical destinations of that transmitted force include the basal cell adhesions to the ECM that are mediated by integrin receptors. Apical fluid shear stress has been long been known to be transferred to the cell's basal focal adhesions [6], and it appears to be transmitted over the actin cytoskeleton [42]. Indeed, competing for immobilized ECM receptor binding with soluble GRGDSP or ligand-mimicking anti-β1 integrin antibody blocked the flow-induced calcium signaling response in our kidney cells, just as completely as cytoskeletal disrupters. Furthermore, the response was ablated as efficiently as if any part of the primary ciliary mechanotransduction pathway was blocked, for example, by using cell culture conditions that prevent cilia formation, cells from polycystin knockout mice, or blocking anti-polycystin antibodies [4].

These observations, however, are complicated by past finding that a subset of polycystin-1 protein can localize to β1-integrin-mediated focal adhesions at the cell base [51]. Although polycystin-1 is not an ion channel, it associates closely with polycystin-2 and both are required for flow-induced calcium signaling in MEK cells [4]. Thus, although we observed no Ca^{2+} response when twisting RGD-coated beads bound to apical β1 integrins, nor did we observe colocalization with cilia, the link between integrins and polycystins deserves further investigation. Another caveat in these studies is that the flow-induced calcium response detectable with our methods also can be inhibited by blocking calcium-induced calcium release from intracellular stores (e.g., using ryanodine or caffeine) [4]. Thus, global changes in cell architecture and mechanics could possibly impact the mechanotransduction process at multiple levels in these cells.

Nevertheless, while not discounting the central role of cilia-associated polycystins in the MEK mechanotransduction response [4], these data suggest a model that is multifactorial, with efficient ciliary force transmission and downstream calcium signaling requiring counterbalanced tension and compression through the cytoskeleton. In this model, integrin-mediated ECM adhesion sites function as stable but dynamic anchors at the cell base that also resist tractional forces generated within the contractile actin cytoskeleton, and thereby modulate the net level of cytoskeletal prestress (isometric tension) in the cell. Cytoskeletal prestress is a critical determinant of cell shape stability; for example, dissipating cytoskeletal tension makes cells more flexible. In the case of MEK cells, decreasing isometric tension in the cell (or disrupting

cytoskeletal filaments in the cytoplasm) may reduce the ability of the primary cilium to maintain its resistance to fluid flow and thus, indirectly influence its mechanosensing function. In the end then, the mechanical signaling response triggered by bending of the primary cilium appears to be governed by a global balance of forces throughout the entire structure of the cell, not just by local membrane processes.

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