Mechanical properties of primary cilia regulate the response to fluid flow

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Rydholm S, Zwartz G, Kowalewski JM, Kamali-Zare P, Frisk T, Brismar H. Mechanical properties of primary cilia regulate the response to fluid flow. Am J Physiol Renal Physiol 298: F1096–F1102, 2010. First published January 20, 2010; doi:10.1152/ajprenal.00657.2009.—The primary cilium is a ubiquitous organelle present on most mammalian cells. Malfunction of the organelle has been associated with various pathological disorders, many of which lead to cystic disorders in liver, pancreas, and kidney. Primary cilia have in kidney epithelial cells been observed to generate intracellular calcium in response to fluid flow, and disruption of proteins involved in this calcium signaling lead to autosomal dominant polycystic kidney disease, implying a direct connection between calcium signaling and cyst formation. It has also been shown that there is a significant lag between the onset of flow and initiation of the calcium signal. The present study focuses on the mechanics of cilium bending and the resulting calcium signal. Visualization of real-time cilium movements in response to different types of applied flow showed that the bending is fast compared with the initiation of calcium increase. Mathematical modeling of cilium and surrounding membrane was performed to deduce the relation between bending and membrane stress. The results showed a delay in stress buildup that was similar to the delay in calcium signal. Our results thus indicate that the delay in calcium response upon cilia bending is caused by mechanical properties of the cell membrane.

NORMAL CELL FUNCTION INVOLVES A COMPLEX ARRAY of interacting cellular processes that take input from external stimuli. One class of external stimuli is the physical factors, including temperature, pressure, stretch, and flow. The primary cilium has been described as an organelle involved in fluid flow sensing. It is ubiquitously present on nearly all mammalian cells, with the exception of cells of myeloid and lymphoid origin (21). It is expressed only once per cell and originates from the distal end of the mother centriole, which migrates near the apical plasma membrane upon cilia formation (22). Structurally, it is composed of nine pairs of circularly arranged microtubules (9 + 0) without dynein arms and is not motile.

A cilium structure is comprised of more than 650 proteins (3) with different functions, such as temperature sensitivity [transient receptor potential vanilloid 4 (TRPV4) (6)], protein binding [polycystin-1; (10)], ion channels [TRPV4, TRRP2 (12)], and protein trafficking (28). When cilia or ciliary signaling molecules are absent or inhibited, various pathological disorders can occur, giving symptoms such as impaired brain development (1), obesity, hypertension, hearing impairment, blindness, diabetes, lack of muscle control (20), or cystic disorders in liver (19) and pancreas (3). However, most of the disorders coupled to primary cilia have one symptom in common, which is renal failure due to kidney cyst formation. Understanding the pathology of these diseases from a molecular or mechanistic level may give insights that can lead us to new treatment strategies.

In 2001, Praetorius and Spring (24) first observed that primary cilia expressed on kidney epithelial cells could generate intracellular calcium in response to fluid flow. Since then, a protein complex between polycystin-1 and polycystin-2 was found to be localized on the cilium (35) and was suggested to function as a calcium channel in the ciliary membrane (18), initiating calcium influx upon ciliation bending. Malfunction of either polycystin-1 or polycystin-2 disrupts the cilia-induced calcium response and leads to autosomal dominant polycystic kidney disease, indicating a direct coupling between the calcium signal and renal cyst formation. Another study showed that polycystin-2 and TRPV4 also colocalize on cilia and form a coupled complex that responds to fluid flow (12). How these molecular sensors detect flow and generate signaling, such as intracellular calcium response, is not fully known. Several studies have also revealed that the flow-induced calcium signal shows a significant lag between the onset of fluid flow and calcium increase (16, 25). This lag differs between cell types, but in Madin-Darby canine kidney (MDCK) and rabbit-collecting duct cells it was found that the peak occurs ~30 s after a stimulus.

To understand how cilia motion is integrated into biological processes, cilia in flow have been studied using fluid dynamical modeling (2, 4, 5, 27). These models describe cilia movement in the presence of forces using biophysical parameters such as length, diameter, bending, and membrane resistance (5). They have shown that with basic cilia parameterization, complex cilia motion and ciliary stress can be described for both motile and nonmotile cilia. What is not known is whether cytosolic motion can directly translate into intracellular signaling, e.g., mediated by calcium, or is incidental, and molecular sensors alone are responsible for fluid flow mechanical signal transduction. There are several hundred proteins that comprise a cilium structure (3), and some of these proteins are sensitive to extracellular changes such as fluid flow. Whether these molecular sensors detect flow and generate signaling aided directly or indirectly by cilium bending is not fully known. Any model that describes an intracellular calcium response due to flow will also need to describe the time delay between the start of fluid flow and when a cell generates a signal (8, 23).

We have modeled ciliary response to bending using basic cilium parameters such as length (8 μm, although in vitro, cilia can grow to lengths of 55 μm) (24), bending rigidity (32, 34), and membrane stiffness. The model describes an elastic cilium surrounded and covered by a viscoelastic membrane that was simulated using finite element methods (FEMs). The simulations show that the actual bending of the cilium is fast, whereas...
the ciliary membrane stress gradually builds up in the vicinity of the cilium’s base and requires ≥20 s before reaching a maximal value after the application of force. We have also measured intracellular calcium signaling from MDCK cells and observed a similar time delay. It was also noted that the longer a force (fluid flow) was applied to cilia the stronger the intracellular calcium signal, which reached a maximum value 30 s after the start of flow.

To deduce the position of cilia stress areas, we observed primary cilia motion in fluid flow using confocal microscopy. We observed that the distal parts of cilia, ~5 μm above the base, move in time with fluid flow but have no effect on intracellular calcium response. The basal part, on the other hand, shows limited deflection upon application of flow. This suggests that the lower part of cilia is the region responsible for flow sensitivity. These data, together with results from the cilia stress model, suggest that the delayed intracellular calcium response to fluid flow is due to the length of time for stress to build up on the cell’s membrane, resulting from cilia bending, before signaling can begin. Since polycystin-1 and -2 and TRPV4 are at least two possible coupled molecular flow sensors that colocalize around the base of cilia, the localization of stress at the base of the ciliary membrane could be a mechanical means of altering the conformation of these sensors. Changes in conformation could lead to opening of calcium channels and signaling pathways.

METHODS

Cell culture. All experiments were performed on MDCK cells (purchased from ECACC). The cells were cultured in modified Eagle’s medium (MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.1 mM nonessential amino acids (Sigma, St. Louis, MO) at 37°C and in an atmosphere containing 5% CO2.

Intracellular calcium. Intracellular calcium (Ca2+) responses were measured using the fluorescent calcium indicator fluo-4 (ex 494 nm/em 516 nm; Invitrogen, Carlsbad, CA). The dye was loaded by 105 cells/ml was seeded onto 28-mm coverslips in 35-mm petri dishes, giving an initial density of 200 cells/mm2, and was then cultured for 3-7 days before experiments.

Intracellular calcium. Intracellular calcium (Ca2+) responses were measured using the fluorescent calcium indicator fluo-4 (ex 494 nm/em 516 nm; Invitrogen, Carlsbad, CA). The dye was loaded by incubation for 30 min at 37°C with 2.5 μM Fluo-4-AM and 5 mM probenecid (Sigma, St. Louis, MO). All experiments were performed using phosphate-buffered saline (PBS), which contained (in mM) 110 NaCl, 4.0 KCl, 1.5 CaCl2, 1.2 MgCl2, 20.0 HEPES, 1.0 NaH2PO4, and 10 n-glucose, and the pH was adjusted to 7.4 at 37°C.

Immunocytochemistry. Fixation and immunocytochemistry were performed using cells cultured on coverslips. Cells were fixed in 4% paraformaldehyde solution containing 0.1% Triton X-100 for 10 min at room temperature. After rinsing with PBS, the cells were stained with a monoclonal mouse antibody against acetylated α-tubulin (Sigma), followed by an anti-mouse antibody labeled with Alexa fluor 488 (Invitrogen). The coverslips were mounted on glass slides with Immumount and kept dark at room temperature for 3 h and stored at 4°C until used.

Transfection. Cilia of living cells were imaged by transfection with cDNA coding for full-length rat dopamine D5 receptor and subcloned into an enhanced green fluorescent protein (GFP) vector. Transfection was performed using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

Cilia lengths. Lengths of cilia from culture day 3 to day 6 were measured by confocal microscopic analysis of cells immunostained for acetylated α-tubulin. Two to four plates from each day were analyzed and revealed that the cilia lengths were 3.5 ± 0.75 μm on day 3, 4.8 ± 0.67 μm on day 4, 6.7 ± 1.6 μm on day 5, and 11.6 ± 2.0 μm at day 6.

Deciliation. Removal of primary cilia was done by supplementing cultured MEM with 4 mM aqueous chloral hydrate (Sigma) for 72 h. The supplemented medium was changed twice daily. After 72 h the cells were washed two times with PBS, and fresh medium was added for 24 h before cells were used in flow experiments.

Microscopy. All imaging of intracellular calcium, transfected, and immunostained cells were performed using confocal microscopy (LSM 510; Carl Zeiss) with a 63x objective. Alexa fluor 488, and GFP fluorescence was detected using 488 nm excitation and a long-pass 505-nm filter. Calcium images were recorded every 5 s for ≤1 h, and the whole image as well as individual cells were analyzed.

Shear. Laminar flow can be generated by different means; in a previous study we used a microfluidic device in silicon (30). Here we have used a different approach. Laminar flow was generated by controlling the rotation of a 2-cm diameter Plexiglas cylinder, which was positioned 200 μm above and parallel to a confluent layer of MDCK cells cultured on a coverslip. Cells were immersed in 900 μl of PBS solution supplemented with probenecid (5 mM) to inhibit the leakage of fluo-4. A schematic of the experimental setup is shown in Fig. 1. Positioning of the cylinder above the cells was aided by 200-μm spacers attached to the end cap of the cylinder. Cylinder rotation was controlled by a DC motor (ELFA Micromotors) and a power supply/function generator (Wavetek).

Cells were exposed to either continuous or pulsate (step function, on and off) flow from 5 s up until 5 min. Several consecutive flow cycles were applied in each experiment, with an ~5-min resting period between each cycle. Cells were imaged in a region 3 mm from the cylinder center, as the flow here can be considered to be linear (not rotating), and there is also enough distance to avoid interference from the spacers. All experiments were performed at 37°C. The flow device uses an open chamber configuration, which eliminates the risk of oxygen deprivation and suffocation. Temperature was maintained using a microscope heating insert, and the cell buffer remained unchanged during the course of an experiment.

The fluid flow field above the ciliated cells can be described by a planar flow model, where the upper and lower boundaries (i.e., the cylinder and the coverslip) have nonslip conditions. If the rotational period of the cylinder is T and the distance from the center of the cylinder to the cells of interest is r, the rotational velocity of the cylinder is

\[
v_{cyl} = \frac{2\pi r}{T}.
\]

The two nonslip boundaries and zero pressure gradient result in a constant velocity gradient described as

\[
\frac{dv}{dz} = \frac{v_{cyl}}{d},
\]

Fig. 1. Left: cross-section of flow setup with coverslip holder and rotating cylinder. Cells are imaged ~3 mm from center of cylinder. Right: schematic drawing of linear flow profile.
where $d = 200 \, \mu m$ is the distance between the cells and the cylinder; see Fig. 1. The shear in the fluid is calculated by

$$\tau = \eta \frac{dv}{dz} = \eta \frac{v_{cy} - v}{d} = \frac{2\pi r}{d},$$

where $\eta$ is the viscosity of the fluid and was taken to be that of water at 37°C. The resulting shear was between 10 and 60 mPa.

**Ca\textsuperscript{2+}** analysis. Calcium responses were quantified as relative change in fluo-4 intensity. Intensity measurements were compared within the same experiment and presented as relative change in response. Each pulse was constructed using the shell and solid structure descriptions in the von Mises definition as

$$\sigma_{\text{mises}} = \sqrt{\sigma_{xx}^2 + \sigma_{yy}^2 + \sigma_{zz}^2 - \sigma_{xy} \sigma_{yx} - \sigma_{xz} \sigma_{zx} + 2\sigma_{xy} \sigma_{xz} + 2\sigma_{yz} + 3\sigma_{zz}^2},$$

where $\sigma_{xx}, \sigma_{yy}, \sigma_{zz}$, and $\sigma_{xy}$ are components of the dimensionless mesh element deformation calculated in the FEM simulation.

A Rayleigh model with stiffness damping as a membrane parameter was included. Lim et al. (15) have listed parameters from references therein using viscoelastic linear solid models. In this kind of model, a time constant, corresponding well within an order of magnitude to a Rayleigh stiffness damping, can be calculated as (31)

$$t_{\text{damp}} = \frac{\eta_m}{k_1 (1 + k_1 / k_2)},$$

where $k_1$ and $k_2$ are elastic constants and $\eta_m$ is the viscosity of a viscoelastic material. The values listed by Lim et al. (15) result in time constants ranging from 0.6 to 150 s. In our simulations Rayleigh stiffness damping was set to 10 s, well within the span of literature values. Other parameters are listed in Table 1.

Simulations were run to calculate the displacement and stress of the system as a function of time with pulsed and continuous flow for $\leq 3$ min. Shear was applied to the axoneme in the form of a horizontal drag force per unit length caused by fluid flow, as published previously by Resnick and Hopfer (26):

$$f_{\text{drag}}(z) = \frac{4 \pi \eta v(z)}{1 - \gamma - \frac{1}{4} \frac{\gamma}{\eta}}$$

at the cylindrical part of the cilium, where $\gamma$ is Euler’s constant and $\rho$ the density of the fluid, and at the semispherical tip of the cilium:

$$f_{\text{drag}}(z) = 3 \pi \eta a v(z).$$

A boundary condition in the model is that the outer edge of the membrane in the cell surface has zero displacement. Stress and displacement in the membrane and inner structure were calculated using the built-in tools in Comsol Multiphysics and the scripting interface between Comsol Multiphysics and Matlab (The Mathworks, Natick, MA).

**RESULTS**

**Modeled delay in membrane stress is similar to measured calcium delay as well as response amplitude.** Modeling of cilium bending and membrane stress was performed in Comsol Multiphysics. According to the model, the rate of stress building up in the cell membrane is significantly slower compared with the actual bending or deflection of the cilium (Fig. 2A). The delay in stress buildup is due predominantly to mechanical membrane properties such as spring constant and damping.

Flow experiments performed on MDCK cells cultured between 3 and 7 days and stained with the calcium-sensitive dye fluo-4 showed similar results. Immunostain for acetylated $\alpha$-tubulin shows that cells at this stage of culture have well-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
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<tr>
<td>$r$</td>
<td>Radius of cylinder in device</td>
<td>3 mm</td>
</tr>
<tr>
<td>$d$</td>
<td>Thickness of fluid layer</td>
<td>0.2 mm</td>
</tr>
<tr>
<td>$T$</td>
<td>Rotational period of device</td>
<td>1.5 s</td>
</tr>
<tr>
<td>$a$</td>
<td>Radius of cilium (32)</td>
<td>100 nm</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Density of fluid</td>
<td>1,000 kg/m\textsuperscript{3}</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity of fluid (water at 37°C)</td>
<td>$6.97 \times 10^{-4}$ Pa s</td>
</tr>
<tr>
<td>$E_I$</td>
<td>Flexural rigidity of cilium (7, 16, 32)</td>
<td>$1.4 \times 10^{-22}$ Nm\textsuperscript{2}</td>
</tr>
<tr>
<td>$l$</td>
<td>Geometrical radius of membrane structure</td>
<td>2 $\mu$m</td>
</tr>
<tr>
<td>$A$</td>
<td>Estimated cross-sectional area of membrane</td>
<td>$10^{-14}$ m\textsuperscript{2}</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Membrane spring constant (17)</td>
<td>$5 \times 10^{-3}$ N/m</td>
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Cilium parameters.
developed cilia (24). Experiments revealed an approximate time delay of 20 s before the calcium signal was initiated (Fig. 2B and Supplemental Video S1; Supplemental Material for this article can be found on the AJPRenal Physiology web site). Furthermore, a single pulse with a duration of 30 s was able to generate a calcium response with equal amplitude as continuous flow (2 min), whereas a single pulse of ≤5 s generated little or no response (Fig. 2C).

Removal of cilia abolishes calcium signal. To verify that flow-induced calcium increase depends on the presence of cilia, deciliation with 4 mM of chloral hydrate for 72 h was performed. This process removed the cilia and abolished the calcium increase in response to flow (Fig. 2B).

Modeled stress buildup due to pulsate flow corresponds well with calcium response. The model predicts that, with multiple short pulses of flow, membrane stress is gradually built up, and at higher frequencies (>0.1 Hz) maximum stress reaches ~60% of the stress compared with continuous flow (Fig. 3A and Supplementary Video S2). In theory, several pulses should thus be able to generate enough stress to trigger a calcium response even when a single pulse does not. Experiments with continuous flow as well as pulsate flow with frequencies ranging from 0.1 to 2 Hz were performed. In each experiment, several consecutive flow cycles were applied (Fig. 3C), and calcium responses were compared between different flow treatments within the same experiment. All experiments included at least two separate flow cycles with continuous flow and two cycles with pulsate flow. Analysis revealed that the cells did respond to multiple flow pulses but with a calcium increase that was significantly lower compared with continuous flow for all frequencies. However, between the various frequencies, no statistically determined differences were found.

To analyze whether the reduced calcium signal depends on fewer responding cells or decreased signal from each cell, 256 areas in each image were analyzed separately with regard to response or no response for each flow cycle. This analysis revealed a statistically significant decrease in both number of responding cells as well as response amplitude with 30–50% for pulsed flow compared with continuous flow (Fig. 3B). Also, there were no significant differences found between the separate frequencies.

Cilium bending is fast compared with calcium signal. To visualize primary cilia of living cells and to perform real-time measurements of deflection rates, MDCK cells were transfected with a dopamine 5 receptor construct conjugated to GFP, which predominantly targeted cilia. Transfected cells were then exposed to continuous, pulsate, or oscillatory flows (forward-backward) with different frequencies and flow rates. Figure 4A shows an en-face view time series of a cilium exposed to 1 Hz frequency oscillating flow with 19 mPa shear. The cilium tip rapidly switches direction in accordance with flow, thus within 0.5 s. For real-time cilia bending, see Supplementary Video S3. Modeled cilium bending shows similar results; displacement of the cilium after application of 44 mPa shear is a process that occurs within seconds (Fig. 2A, thin curve).

To examine whether prolonged flow led to a gradual increase in bending, three-dimensional imaging of cells exposed to continuous flow from 0 to 5 min was performed. No difference in amount of bending was possible to detect, indicating that cilia rapidly reach a maximal deflection that does not change with time. Slow bending of cilia is thus not likely to explain the time delay for calcium signaling.

Membrane stress is likely localized near the base of the cilium. Figure 5B shows the color-coded stress as modeled in the ciliary membrane (left and middle) and the microtubular structure (right). In the membrane, the stress is located distinctly near the base of the cilium (Fig. 5B, left and middle).
The low stress in the rest of the ciliary membrane is likely due to the tight connection between the membrane and the more rigid microtubules, which will take up the largest part of the applied force. The stress in the microtubules is more spread, albeit still localized toward the cilium’s base. These results indicate that it is the lower part of the cilium that is most likely to be involved in calcium signaling.

Figure 4B shows z sections of a cilium bending in response to increasing levels of continuous shear from no flow up to 55 mPa. The figure shows that the top part of the cilium is increasingly bent with higher force, whereas the lowest part seems to remain in almost the same tilted angle as it had before any flow was applied. Comparing this bending to the modeled bending (Fig. 5), where the cilium has the same flexural rigidity along the entire length, suggests that there is a difference in rigidity between the lower 3–5 μm and the top of the cilium. This effect is seen in most cilia we have studied and is more pronounced in longer cilia (Fig. 4B, right). This also points in the direction that the basal part is able to take up most stress.

Slow frequency flow pulses generate multiple calcium peaks. One single flow pulse with 30 s duration was able to generate
maximum calcium response. Thus several pulses with a frequency of 0.017 Hz (cycle time 60 s) were tested. As shown in Fig. 6, cells responded by generating multiple peaks according to the applied flow pulsations.

DISCUSSION

It is well described in the literature that primary cilia have a flow-sensing function (24). Several studies have focused on the molecular mechanisms involved in cilia calcium signals as well as the location of the proteins involved (18). Those studies suggest protein complexes located near the base of the cilium and different mechanisms for stretch-activated calcium channel functions. However, little attention has been given to the mechanical properties of cilia and how a fluid flow field can affect the structure to transduce from a mechanical to a chemical signal.

We propose here that the structure of primary cilia, with a relatively rigid central structure of microtubule bundles covered by an elastic cell membrane, has important dynamic mechanical properties that serve to decode slow fluctuations in fluid flow. Our experiments and simulations show that the primary cilia can function as a low-pass filter, where fast fluctuations in fluid flow do not result in any signals, whereas slower and steadier flows initiate intracellular calcium signals. Furthermore, our data point out that the region where maximal membrane stress occurs is at the base of the cilium, where it has been shown that the relevant protein complexes for calcium signaling are located. The length of the cilium has an important function; by reaching out into the lumen it becomes more sensitive to absolute flow, and edge effects from cell surfaces are reduced.

For the physiological function of a low-pass filtering in flow sensitivity, one can so far only speculate. A possible function could be in avoiding spurious signaling due to fluctuations not resulting in any net flow. Normal renal function requires that the flow through the nephron is kept within a narrow range. If it is not, renal ability to maintain bodily solute and water balance will be compromised. A mean shear stress during a certain time can be translated into transport of a minimum volume of liquid through the nephron. The calcium signaling would thus verify that renal...
fluorid transport does not fall below a critical value. Such signaling could provide a continuous feedback of nephron flow conditions and participate in the regulation and fine tuning of secretion and absorption processes.

Interestingly, the delay in calcium signaling is within the same time domain as the oscillations in the tubuloglomerular feedback (TGF), a mechanism in macula densa that regulates glomerular filtration rate (9, 13). Studies using in vivo multiphoton microscopy (11, 29) have revealed that TGF-mediated oscillations occur with a cycle time of 20–50 s and that similar periodicity in tubular flow rates occur with a delay of 5–10 s in proximal tubule and 25–30 s in distal tubule. The delay in calcium signal is thus within the same order of magnitude as the inherent periodicity of normal nephronic flow pulsations, pulsations that are detectable throughout the nephron.

Our results showed that pulsed flow with a cycle time of 60 s can generate regular calcium peaks in ciliated cells. Thus one could speculate that regular calcium peaks also occur in vivo in response to TGF-mediated pulses. Calcium peaks are known to activate various signaling pathways and transcription factors, depending on the peak frequency. For example, slow calcium oscillations (3–5 min between peaks) have been shown to activate the transcription factor NF-κB, which in the developing kidney leads to increased cell proliferation and protection from apoptosis (14).

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DISCLOSURES

No conflicts of interest are declared by the authors.

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