J. Membrane Biol. 184, 71–79 (2001)
DOI: 10.1007/s00232-001-0075-4

The Journal of
Membrane
Biology

Bending the MDCK Cell Primary Cilium Increases Intracellular Calcium

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Received: 4 April 2001/Revised: 28 June 2001

Abstract. We tested the hypothesis that the primary cilium of renal epithelia is mechanically sensitive and serves as a flow sensor in MDCK cells using differential interference contrast and fluorescence microscopy. Bending the cilium, either by suction with a micropipette or by increasing the flow rate of perfusate, causes intracellular calcium to substantially increase as indicated by the fluorescent indicator, Fluo-4. This calcium signal is initiated by Ca^{2+}-influx through mechanically sensitive channels that probably reside in the cilium or its base. The influx is followed by calcium release from IP_3-sensitive stores. The calcium signal then spreads as a wave from the perturbed cell to its neighbors by diffusion of a second messenger through gap junctions. This spreading of the calcium wave points to flow sensing as a coordinated event within the tissue, rather than an isolated phenomenon in a single cell. Measurement of the membrane potential difference by microelectrode during perfusate flow reveals a profound hyperpolarization during the period of elevated intracellular calcium. We conclude that the primary cilium in MDCK cells is mechanically sensitive and responds to flow by greatly increasing intracellular calcium.

Key words: Flow — Calcium-induced calcium release — Mechanical sensitivity — IP_3 — Gap junction — Gadolinium

Introduction

The primary cilium, a solitary nonmotile structure projecting from the centriole of mammalian cells, has been a subject of interest for anatomists for over a century (Zimmermann, 1898). Surprisingly, its function in mammalian cells is completely unknown. All of the epithelia of the mammalian kidney, with the exception of the intercalated cells of the collecting duct, express a single primary cilium on their apical (lumenal) surface (Latta, Maunsbach & Madden, 1961; Pfaller & Klima, 1976). Physiologists have paid scant attention to these structures until recently when the mechanical properties of the primary cilia of cultured cells of renal origin were measured and it was proposed that they could serve as flow sensors (Schwartz et al., 1997). This proposal was based on the observation that flow rates comparable to those observed in renal tubules (10–15 nl/min or 15–75 μm/sec) resulted in a deflection of the cilium of a few microns and bent the shaft of the cilium to an extent commensurate with stretch-activated mechanosensing.

The primary cilia of renal tubular cells are generally about 2–3 μm long, whereas those of cultured renal cells become much longer as the cells age and may extend 50 μm or more into the medium (Roth, Rieder & Bowser, 1988). MDCK cells, a widely used cultured cell line derived from the collecting duct of the canine kidney, exhibit two cell types analogous to the principal and intercalated cells of the collecting duct (Rindler, Chuman, Shafter & Saier, 1979). MDCK principal cells express a primary cilium that is visible by high-resolution differential interference contrast (DIC) microscopy. About one week after splitting, the primary cilium of MDCK principal cells is about 8 μm long and is well suited for testing the hypothesis that it serves as a mechanosensor of fluid flow.

Materials and Methods

Cell Culture

MDCK cells (passages 62–76 from the American Type Culture Collection, Rockville, MD) were grown to confluence on 25-mm diameter cover slips in Dulbecco's modified Eagle medium (DMEM) with 10%

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fetal bovine serum (Gibco, Grand Island, NY) and 2 mM glutamine, but without riboflavin, antibiotics or phenol red, as previously described (Xia et al, 1998).

Microscopy and Perfusion

The cells were first incubated for 15 minutes with the calcium-sensitive probe Fluo-4-AM (5 μM) at 37°C, washed to remove excess probe and allowed a 10-minute de-esterification period. Then they were placed in a custom-designed laminar-flow perfusion chamber on the stage of an inverted microscope (Diaphot, Nikon, Melville, NY) equipped with differential interference contrast (DIC) combined with low light level fluorescence as described previously (Xia et al., 1998). Imaging was performed with a 100×/1.3 N.A. lens (Nikon) and an intensified CCD camera (ICCD-1001, Video Scope, Sterling, VA). Fluorescence excitation at 477 nm was supplied by an argon laser, and emission was detected at 520 nm or longer. The perfusion chamber had a 16-mm long flow path that was 3 mm deep and 3 mm wide. The top of the perfusion chamber was made from half of a 25-mm coverslip positioned appropriately for high-resolution microscopy of the cells immediately beneath. Flow rates were calibrated by measurement of the efflux into a reservoir of known volume; a rate of 1 μl/sec corresponds to a linear velocity of 11 μm/sec, equivalent to a tubular flow rate of 7 nl/min. The cilia were visualized by DIC, and the fluorescence was sampled at rate of 0.5 Hz during manipulation of a primary cilium. Fluo-4 is a single-wavelength excitation probe and the intensity of emitted fluorescence can be influenced by changes in cell volume or thickness. To control for cell-volume or optical-path changes, some preparations were loaded with a pH-sensitive dye (BCECF-AM) and fluorescence was excited at 458 and 488 nm as previously described (Chatton & Spring, 1995). Fluorescence was measured in a region of interest near the base of the cilium and was expressed relative to the baseline value, chosen as the mean of 5 intensity observations prior to experimental manipulation, for comparison with the mean of 5 values around the peak response. After the measurements were completed, the responsiveness of the system was checked by treatment of the cells with ionomycin (10 μM), which resulted in a relative increase of the fluorescence response of 1.59 ± 0.05 fold ($n = 33$). All experiments were carried out at 37°C, pH 7.4. The opening in the top coverglass of the perfusion chamber enabled the introduction of a micropipette that was attached to a pressure control system and mounted on a motorized micromanipulator. The pipette tip was brought close to the cilium tip, but always at least 4 μm above the apical membrane (*see* Figure 2*A*). Application of a slight negative pressure caused the tip of the cilium to bend towards the pipette. The cilium remained bent until the fluorescence measurements were completed. The fluorescence measurements were initiated 20 seconds prior to the movement of the primary cilium. During these experiments the cells were under constant slow perfusion of approximately, 1.5 μl sec^{-1}. In other experiments, the primary cilium was bent by increasing the flow of perfusate to a rate of approximately 8 μl sec^{-1} after the flow had been stopped for a period of 10 minutes.

The perfusion solution had the following composition, in mM: [Na^+] 137, [K^+] 5.3, [Ca^{2+}] 1.8, [Mg^{2+}] 0.8, [C^-] 126.9, [SO_4^{2-}] 0.8, HEPES 14, glucose 5.6, probenecid 5, pH 7.4 (37°C, 300 mOsmol). Ca^{2+}-free solution contained, in mM: [Na^+] 139, [K^+] 5.3, [Mg^{2+}] 0.8, [Cl^-] 128, [SO_4^{2-}] 0.8, HEPES 14, glucose 5.6, probenecid 5, EGTA 1 mM, pH 7.4 (37°C, 300 mOsmol).

Immunofluorescence

MDCK cells, grown on glass coverslips for 4–8 days, were washed twice in phosphate buffered saline (PBS) and fixed for 15 minutes in 2.5% formaldehyde at room temperature. Then the cells were washed twice and permeabilized with 0.3% Triton-X-100 PBS containing bovine serum (15 mg/ml) for 15 minutes. They were then incubated overnight with the primary antibody, anti-bovine α-tubulin mouse monoclonal (1 μg/ml) at 4°C. After thorough washing, the cells were incubated for 3 hours with the secondary antibody, FITC-conjugated anti-mouse IgG (dilution 1/100) at room temperature. The cells were washed again and mounted in an anti-fade solution for observation with a confocal microscope (Odyssey, Noran Inst., Middleton, WI).

Electrophysiology

Glass microelectrodes with a tip diameter of <1 μm were filled with 3 M KCl, mounted on a hydraulic micromanipulator (Model MMO-220A, Narishige, East Meadow, NY) and connected to a high-impedance electrometer (Model IE-251A, Warner Instruments, Hamden, CT) for measurement of the apical membrane potential difference (*PD*). Electrode input impedance was monitored continuously, and the cell puncture was monitored by high resolution DIC microscopy.

Materials

Sources of chemicals were: Fluo-4-AM, BCECF-AM, anti-bovine α-tubulin (Molecular Probes, Eugene, OR); verapamil, EGTA, probenecid, ionomycin, ryanodine, thapsigargin, nitrendipine, Gd^{3+}, anti-mouse IgG FITC conjugate (Sigma, St. Louis, MO), ω-conotoxin, sFTX-3.3 (Almone Labs, Jerusalem, Israel).

Statistics

All values are shown as mean ± SEM. Statistical significance was determined using the nonparametric Mann-Whitney test; *p* values less than 0.05 were considered significant.

Results

Morphology

The primary cilia of MDCK cells 6 days after splitting are about 8 μm long and exhibit a prominent base at their insertion into the cell membrane and a swelling near their tip (Fig. 1*A*). Normally, these organelles are difficult to see, even with high-magnification DIC, as the cilium extends out of the apical membrane along the optical axis of the microscope image. The DIC image in Fig. 1*A* is obtained by placing a coverglass on the apical surface of the cells and by minimizing the thickness of the intervening fluid layer to bend the cilium. Since the primary cilium contains bundles of microtubules arranged in the "9 + 0" configuration, it can be more readily visualized in a fixed preparation by immunofluorescence with fluorescently labeled anti-tubulin antibodies (Jensen, Jensen & Rieder, 1979). Figure 1*B* shows a confocal microscope image of such a preparation in MDCK cells with the focus adjusted to the plane of the cilium. Figure 1*C* shows a similar image of the apical surface of MDCK cells 4 days after splitting, at which time they do not express a primary cilium.

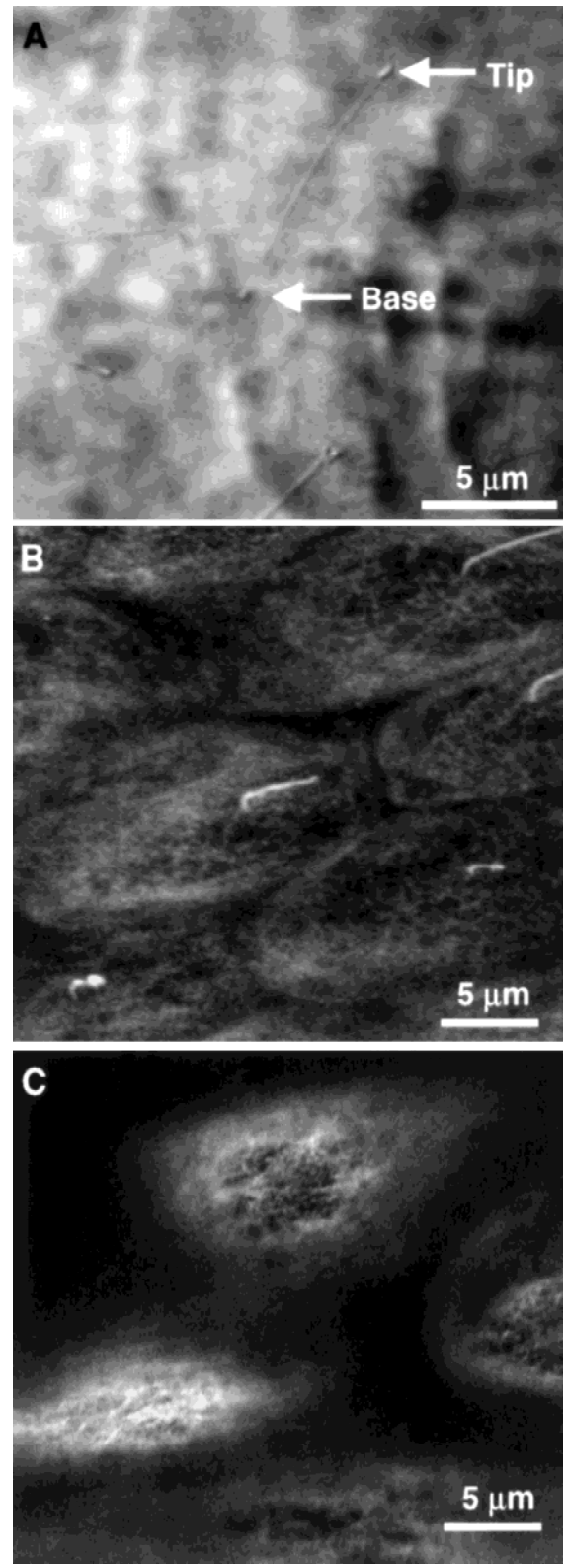

Fig. 1. Light microscopic images of MDCK primary cilia. (*A*) High magnification DIC image (100×/1.32 NA) of cilia (5–8 μm long) that have been bent by a coverslip to make their entire length visible. The site of insertion of the cilium through the cell membrane (the base) and the swelling at or near the tip are indicated. (*B*) Images of fluorescent cilia (3–8 μm long) that have been stained with anti-tubulin antibodies, bent by a coverslip as in panel *A*, and visualized in a confocal microscope with a 100×/1.32 NA objective lens. (*C*) Confocal fluorescence images of young cells (4 days after splitting) that were prepared as in panel *B*, but do not exhibit primary cilia.

Calcium Response to Bending

MDCK cells respond to bending of the primary cilium by a pipette (Fig. 2*A*) with a substantial increase in Fluo-4 fluorescence and, hence, intracellular Ca^{2+} concentration. The thick line in Fig. 2*B* shows the relative change in the intracellular Fluo-4 intensity as the tip of the primary cilium is displaced about 4 μm (mean 3.7 ± 0.1 μm, $n = 143$) by the suction pipette. To avoid direct contact with the cell surface, the pipette is always positioned at least 4 μm above the cell membrane and the suction is maintained so that the tip of the cilium remains bent toward the pipette during the entire measurement period. The fluorescence increases 1.60 ± 0.07 fold ($p < 0.01$, $n = 32$) in response to the movement. The response to bending develops slowly and the time from the initial movement of the cilium to maximal calcium response is 36.7 ± 3.0 sec ($n = 31$). The increased calcium spreads as a wave to the immediately adjacent cells after a delay proportional to the distance from the stimulated cell (thin lines in Fig. 2*B*).

Control experiments with cells loaded with the pH-sensitive dye BCECF do not show any change in intracellular pH or in the amplitude of the fluorescence signals when the cilium is bent either by micropipette ($n = 3$) or by perfusate flow ($n = 43$). This shows that the observed changes in Fluo-4 fluorescence are not due to changes in cell thickness, volume or intracellular pH. Other possible artifacts such as bleaching or dye leakage will tend to diminish the Fluo-4 fluorescence and can not be the cause of the large increase that is observed.

Calcium Response to Flow

Changing the rate of laminar flow on the apical side of MDCK cells produces a similar-magnitude deflection of the cilium and a calcium response to bending (Fig. 2*C*). Upon changing the flow from 0 to 8 μl sec^{-1}, the relative fluorescence increases 1.74 ± 0.07 fold ($p < 0.01$, $n = 37$). The calcium response exhibits considerable lag and is maximal 51.7 ± 8.0 sec ($n = 12$) after initiation of flow. The calcium remains elevated above baseline for a period of 4.6 ± 0.7 minutes ($n = 22$) after the peak is obtained. If the flow is discontinued when the Ca^{2+} re-

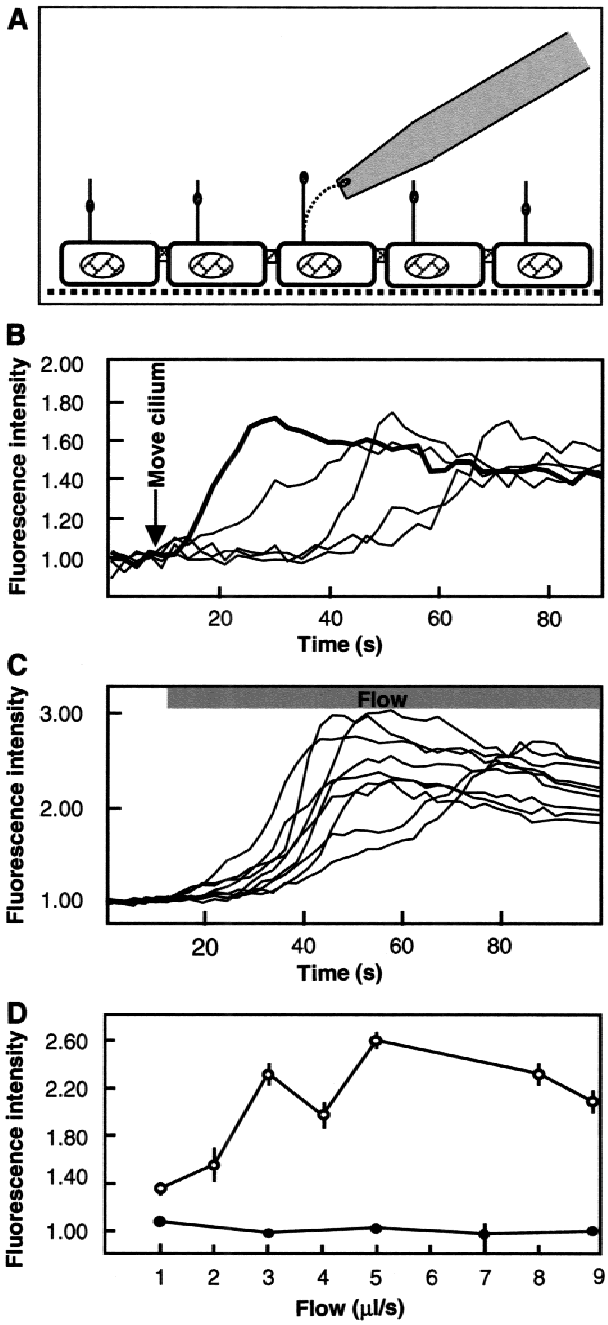

Fig. 2. Changes in intracellular calcium in response to bending of the primary cilium. (*A*) Experimental design for micropipette manipulation of the primary cilium. (*B*) Changes in relative Fluo-4 fluorescence during bending of the primary cilium. The increase is observed first in the cell to which the cilium is attached (thick line) followed by the four immediately adjacent cells (thin lines). (*C*) Changes in the relative fluorescence of nine neighboring cells caused by flow-induced bending of the primary cilia. Initially, the flow was stopped and the fluorescence recorded, then flow was begun at a rate of 8 μl sec^{-1} as indicated. (*D*) The maximal calcium response for various flow velocities. After discontinuation of flow for 30 minutes, the flow was resumed at rates as indicated on the abscissa. The open circles show the maximal increase in fluorescence intensity relative to baseline in confluent MDCK cells; all values represent a significant increase above baseline ($p < 0.01$). The filled circles represent the lack of response of subconfluent MDCK cells that do not exhibit primary cilia. The bars indicate SEM. The number of tested cells are for the confluent: 8 (1 μl sec^{-1}), 9 (2 μl sec^{-1}), 20 (3 μl sec^{-1}), 10 (4 μl sec^{-1}), 11 (5 μl sec^{-1}), 15 (8 μl sec^{-1}), 13 (9 μl sec^{-1}), and for the nonconfluent: 8 (1 μl sec^{-1}), 6 (3 μl sec^{-1}), 6 (5 μl sec^{-1}), 6 (7 μl sec^{-1}), 6 (9 μl sec^{-1}).

sponse is maximal, the cells can be re-stimulated after the intracellular Ca^{2+} returns to the baseline. However, a recovery period of 25 minutes is needed for the second response to be of an amplitude equal to the first. The amplitude of the flow-induced Ca^{2+} signal is dependent on the flow rate (Fig. 2*D*). Intracellular calcium is significantly increased above baseline at a flow rate of 1 μl sec^{-1}, and at 3 μl sec^{-1} or higher the cells develop a maximal response. The closed circles in Fig. 2*D* represent records from subconfluent MDCK cells (day 4 after splitting) that lack the ability to respond to flow. Close inspection of day-4 MDCK cells, both by DIC and immunofluorescence microscopy, revealed that they do not exhibit primary cilia (*compare* Figs. 1*B* and 1*C*).

Calcium Dependence

The bending-induced Ca^{2+} response is dependent on extracellular calcium. Changing the perfusate to a Ca^{2+}-free solution (containing 1 mM EGTA) for at least minutes prior to the measurements completely inhibits the response (Fig. 3*A*). The relative fluorescence is 0.95 ± 0.05 ($p < 0.01$ *vs.* control, $n = 11$), 35 sec after bending the primary cilium, a time at which the response should be maximal. The flow-induced calcium response also requires extracellular calcium (Fig. 3*B*). Changing the perfusate to a Ca^{2+}-free solution (containing 1 mM EGTA) for at least 10 minutes prior to the measurements abolishes the response. The relative fluorescence is 1.08 ± 0.06 ($p < 0.01$ vs. control, $n = 13$), 50 sec after initiation of flow, corresponding to the time of the expected peak calcium response.

Two inhibitors of L type calcium channels—verapamil (10 μM) and nitrendipine (10 μM)—in the presence of extracellular calcium (1.8 mM), fail to influence the response to bending the cilium (Fig. 3*A*). Furthermore, none of the tested calcium channel blockers—verapamil; ω-conotoxin, a blocker of N-type calcium channels; and sFTX-3.3, a blocker of P, Q and T type calcium channels—has any effect on the flow-induced calcium response (Fig. 3*B*). However, the addition of Gd^{3+}, an inhibitor of stretch-activated cation channels, in the presence of calcium completely abolishes both the micropipette- and flow-induced calcium signals. In the presence of 30 μM Gd^{3+}, the relative fluorescence is 1.05

± 0.03 ($p < 0.01$ *vs.* control, $n = 14$), 35 sec after bending the primary cilium (Fig. 3*A*) and 0.99 ± 0.02 ($p < 0.01$ *vs.* control, $n = 33$), 50 sec after the initiation of flow (Fig. 3*B*).

Evidence for Calcium-Induced Calcium Release

Several experiments were performed to determine whether the calcium signal arises only from calcium influx or is a consequence of Ca^{2+}-induced Ca^{2+} release. Removing extracellular calcium after the initiation of the response does not significantly alter the magnitude or time course of the increase or the spread to adjacent cells (Fig. 4*A*). Since this is consistent with the initial calcium influx inducing Ca^{2+} release from intracellular stores, the sensitivity of the response to the depletion of calcium stores by the traditional inhibitors, ryanodine or thapsigargin, was tested. As shown in the Table, exposure to 1 μM thapsigargin for ten minutes in the absence of calcium to deplete the intracellular calcium stores, followed by the reintroduction of calcium in the presence of 1 μM thapsigargin, significantly reduces the response to bending the cilium or flow. In contrast, 10 μM ryanodine is without effect either on the micropipette- or the flow-induced calcium increase. Furthermore, the phospholipase C (PLC) inhibitor U73122 (20 μM) completely abolishes the bending-induced Ca^{2+} signal. Therefore, the calcium response requires both calcium influx and PLC-dependent release of intracellular calcium stores, probably as a result of IP_3 generation.

Spread of the Calcium Response

The bending-induced calcium response spreads, after a delay, to neighboring cells in 22 out of 30 experiments. MDCK cells are known to have gap junctions as they exhibit characteristic ionic coupling and cell-cell solute transfer (Giaume, Sahuquillo, Louvard & Korn, 1986; Ledbetter, Young & Wright, 1986) as well as respond to some agonists with synchronized calcium waves that are sensitive to gap junction blockade by octanol (Rottingen, Camerer, Mathiesen, Prydz & Iversen, 1997). Heptanol, an inhibitor of permeation through gap junctions, significantly reduces the spread of the wave of Ca^{2+} increase (Fig. 4*B*). In the presence of heptanol (3.5 mM), bending of the cilium produces a lateral calcium increase in only 5 out of 20 experiments, and in 4 of these cases the wave spreads to just one other cell ($p < 0.01$ *vs.* control). The magnitude of the calcium response in the cell to which the cilium is attached is not significantly affected by heptanol (1.48 ± 0.09, $n = 20$), supporting the conclusion that heptanol does not interfere with calcium influx or the subsequent release of calcium from stores.

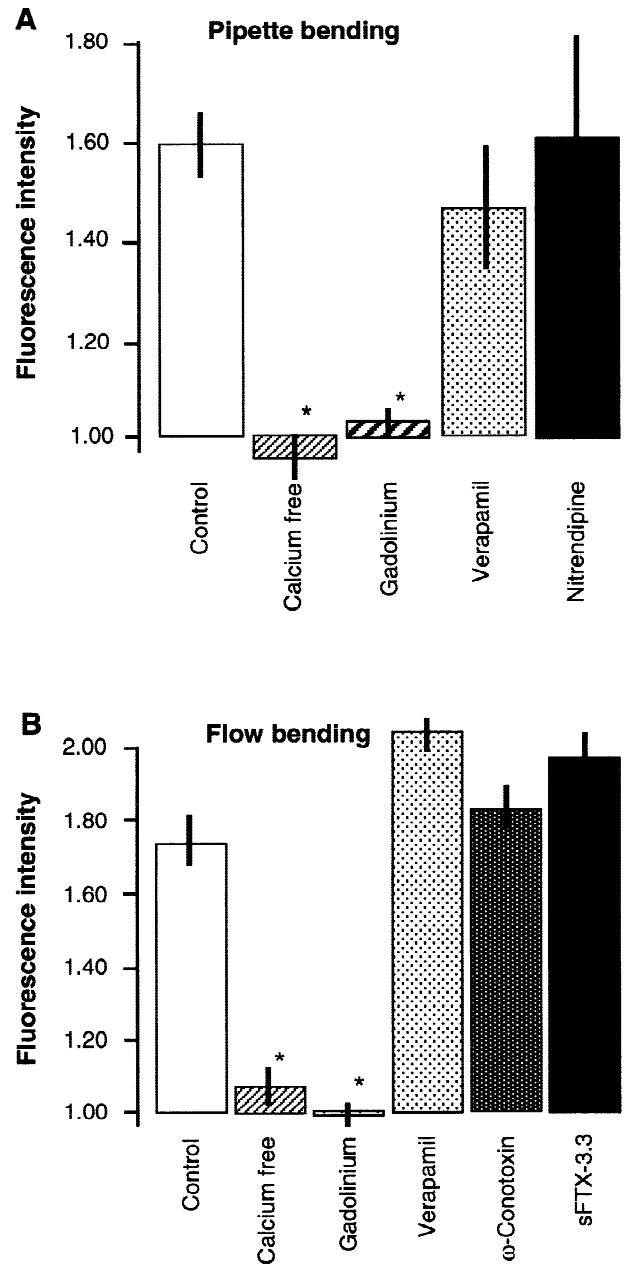

Fig. 3. The effect of extracellular calcium and calcium channel blockers on the calcium signal induced by bending of the primary cilium. (*A*) The relative fluorescence following bending of a primary cilium by pipette. Ca^{2+}-free solution was perfused or the inhibitors, verapamil (10 μM), nitrendipine (10 μM) and Gd^{3+} (30 μM) were added at least 10 minutes before the bending of the primary cilium. The bars represent mean values ± SEM. Significant difference from control at $p < 0.01$ is indicated by an asterisk. The number of experiments are: Control, $n = 32$; Ca^{2+} free, $n = 16$; Gd^{3+}, $n = 15$; verapamil, $n = 7$; nitrendipine, $n = 9$. (*B*) The increase in relative fluorescence following bending of a primary cilium by flow. Ca^{2+}-free solution, Gd^{3+} (30 μM), verapamil (10 μM), ω-conotoxin (1 μM) or sFTX-3.3 (10 μM) were present throughout the experiment from the cessation of the flow to its re-establishment (8 μl sec^{-1}). The number of experiments are: Control, $n = 37$; Ca^{2+}-free, $n = 13$; Gd^{3+}, $n = 33$; verapamil, $n = 7$; ω-conotoxin, $n = 44$; sFTX-3.3, $n = 25$.

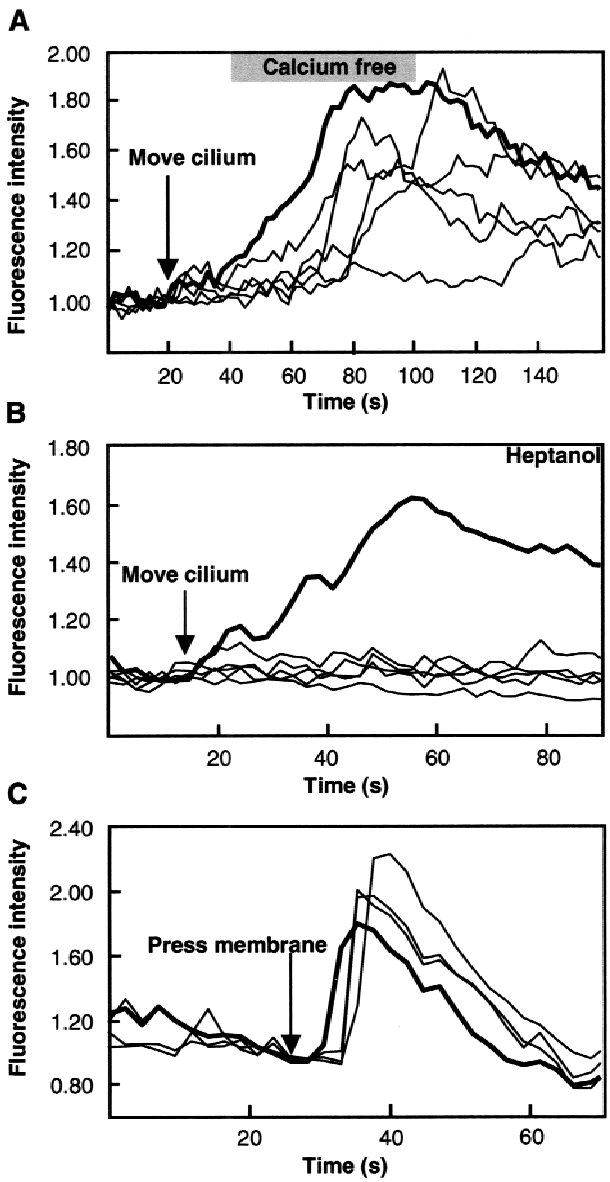

Fig. 4. Demonstration of calcium-induced calcium release, subsequent spread through gap junctions, and the effect of pressing directly on the cell membrane. (*A*) The effect of switching to a Ca^{2+} free perfusate 20 seconds (box) after bending of the primary cilium; 1.8 mM Ca^{2+} was reintroduced after 60 seconds. (*B*) The effect of heptanol (3.5 mM) on the lateral spread induced by bending of the primary cilium. Heptanol was added at least 2 minutes before the experiment. (*C*) Changes in the relative fluorescence from a central region of interest during pressing of the apical membrane with a micropipette. The increase is observed first in the cell whose membrane is pressed (thick line) followed by an increment in the immediately adjacent neighboring cells (thin lines).

Direct Stimulation of the Cell

As previously reported (Hirose et al., 1999), direct mechanical stimulation of the apical membrane of MDCK cells by depressing it with a micropipette results in a 1.90 ± 0.15 fold ($n = 10$) transient increase of calcium (Fig. 4*C*), with a delay from stimulation to maximal calcium response of 9.8 ± 2.7 sec ($n = 10$). This calcium response differs from that induced by bending the cilium, both in its larger amplitude and shorter time delay ($p < 0.05$ and $p < 0.0001$, respectively). Furthermore, the calcium transient is not affected by removal of extracellular Ca^{2+} for ten minutes prior to and during the experiment (amplitude 1.91 ± 0.12, $n = 10$). Therefore, we conclude that the response to contacting the membrane with a micropipette liberates calcium from intracellular stores by a mechanism that does not involve calcium entry across the cell membrane. This result supports the conclusion that the calcium influx caused by bending the cilium does not occur because the cilium deforms the cell membrane and that the mechanically sensitive channels reside in the cilium itself or in its base.

Heptanol also inhibits the lateral spread of increased calcium induced by pressing the apical membrane. In 5 out of 9 control experiments, pressing the apical membrane results in spread to neighboring cells. None of the heptanol-treated preparations ($n = 7$) exhibit lateral spread. In the presence of heptanol, the calcium increase in the mechanically stimulated cell is 2.81 ± 0.37 fold ($n = 6$), significantly larger than control ($p < 0.05$). U73122 (20 μM) also abolishes the lateral spread induced by pressing the apical membrane. Lateral spread of the calcium signal does not occur in any of the experiments in which U73122 is present. U73122 significantly reduces the amplitude of the calcium transient upon pressing the membrane to 1.5 ± 0.07 fold ($p < 0.05$, $n = 11$). We conclude that heptanol blockade of lateral spread prevents messenger egress to the adjacent cells through the gap junctions, while U73122 inhibition blocks the spread because it diminishes messenger generation in the stimulated cell.

Table. Effect of inhibitors on the response to bending or flow

Inhibitor	Concentration μM	Relative Fluorescence (Micropipette)	Relative Fluorescence (Flow)
Thapsigargin	1	1.13 ± 0.03 (16)*	1.23 ± 0.04 (18)*
Ryanodine	10	1.58 ± 0.26 (7)	1.59 ± 0.25 (12)
U73122	20	1.03 ± 0.15 (18)*	—

Number of observations in parentheses. Significant difference from control response at $p < 0.01$ is indicated by an asterisk.

Flow Results in Hyperpolarization of the Apical Membrane *PD*

The large calcium increase that follows bending the primary cilium should open calcium-activated ion channels in the cell membranes. Figure 5*A* shows that the apical

membrane potential difference (*PD*) averages −22.3 ± 2.7 mV in 24 undisturbed cells and hyperpolarizes substantially to −56.0 ± 3.3 mV, peaking 94.2 ± 10.9 seconds after perfusate flow begins. Figure 5*B* shows a typical record of this transient hyperpolarization. After several additional minutes of perfusion, the *PD* spontaneously returns to −22.1 ± 2.6 mV, a value indistinguishable from that in the undisturbed cells. The peak hyperpolarization achieved is consistent with the transient opening of calcium-activated potassium channels and the resultant movement of the *PD* toward the potassium equilibrium potential. The time course of the return of the *PD* to baseline despite the continuous perfusion is consistent with the observed fall in intracellular calcium.

Because puncturing the cell might stimulate the cell in a manner similar to that observed when it is touched by a micropipette, intracellular calcium was monitored during microelectrode insertion. When preparations loaded with Fluo-4 to monitor intracellular calcium are punctured with a microelectrode, there is often a transient increase in intracellular calcium similar to that described above when the apical surface is depressed with a micropipette. Calcium returns to baseline within two minutes after insertion of the microelectrode. Often, instabilities in the *PD* are observed during this period, as well. Therefore, the *PD* values shown in Fig. 5 represent measurements obtained after the elapse of a two-minute or longer recovery period.

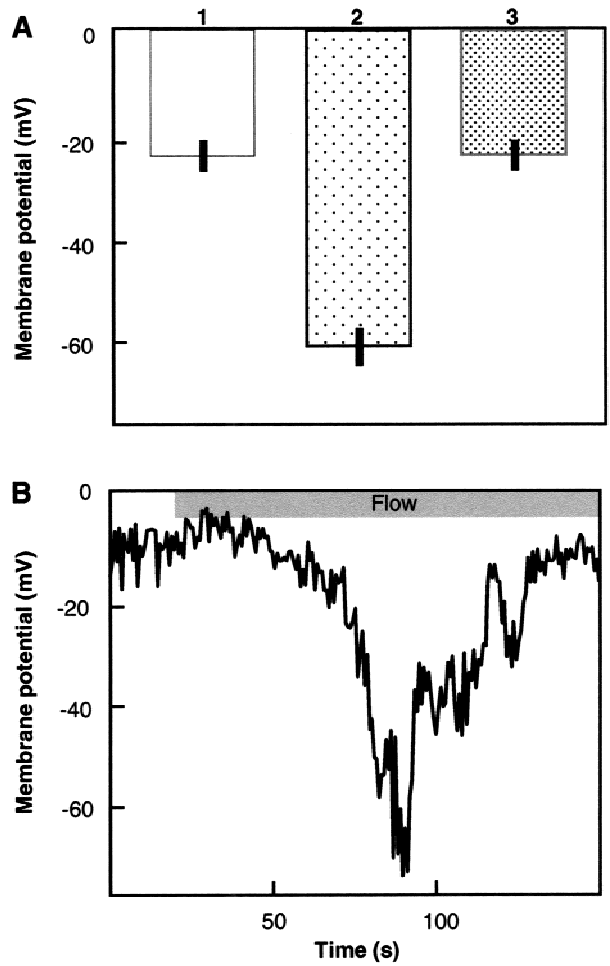

Fig. 5. The apical membrane hyperpolarizes in response to flow. (*A*) The bars show the mean ± SEM membrane potential before initiation of flow (*1*), during maximal hyperpolarization in response to flow (*2*), and during repolarization (*3*). Measurements were initiated 1–2 minutes after the puncture of the apical membrane; $n = 24$. (*B*) A representative trace of the membrane potential measurements with the period of flow indicated by the bar.

Discussion

Our experiments show that this forgotten organelle, the primary cilium, responds to bending with calcium entry through mechanically sensitive channels, followed, after a remarkable delay, by calcium-induced calcium release from intracellular stores. The increase in intracellular calcium subsequently spreads to the neighboring cells, probably by the diffusion of IP_3 and other messengers through the gap junctions. The spreading wave of increased calcium points to a coordinated event within the tissue, rather than an isolated phenomenon in a single cell. The large increase in cell calcium that results may activate calcium-sensitive channels or processes in the cell with profound functional consequences.

Bending the primary cilium of MDCK cells by micropipette or flow produces an increase in the intracellular calcium concentration that is absolutely dependent on extracellular calcium, i.e., calcium influx is obligatory for the occurrence of the signal. The removal of extracellular calcium for 10 minutes or more does not deplete the internal calcium stores because the large calcium increase that occurs in response to direct mechanical stimulation of the cell membrane by contact with a micropipette is unaffected. Since the response to bending the cilium in the presence of calcium is abolished by gadolinium but unaffected by blockage of L, N, P, Q and T type calcium channels, calcium is likely to permeate through stretch-activated, nonselective cation channels such as those previously proposed in MDCK cells (Rothstein & Mack, 1992). Flow-induced increases in the intracellular calcium concentration of A6 cells of similar magnitude and time course were previously reported by Kawahara and Matsuzaki (1992), who speculated that shear forces were the basis for the phenomenon.

The initial calcium influx results in the gradual development of Ca^{2+}-induced Ca^{2+} release from stores. This is evident, since the calcium signal is unchanged by removal of extracellular calcium after bending of the cilium is initiated. Furthermore, the calcium signal is significantly reduced when the intracellular calcium stores are depleted by thapsigargin. The calcium stores

involved are most likely IP_3-sensitive, since the PLC inhibitor U73122 diminishes the response, whereas ryanodine, which at a concentration of 10 μM acts as ryanodine-receptor antagonist, leaves the response unaffected. The slow time course of the calcium transient is remarkable, both for the considerable delay that occurs before any detectable calcium increase and for the long duration of the elevated calcium.

MDCK cells are known to produce an intracellular calcium increase when the apical membrane is depressed (Hirose et al., 1999). This differs from the response produced by bending the cilium since pressing the apical membrane results in a calcium transient that is independent of extracellular calcium and has a completely different time course. We also observe a comparable calcium transient when a microelectrode punctures the cell membrane and speculate that the stimulus could be the result of ATP or UTP release, as has been suggested for airway epithelia (Lazarowski & Boucher, 2001). To prevent inadvertent mechanical stimulation of the apical membrane when bending the cilium, the micropipette was always placed several microns above the apical membrane. We conclude from the differences in these two responses that the channels opened by bending the cilium are located in the cilium or near the cilium base, and not in the apical membrane.

The calcium signal induced by pressing the apical membrane of MDCK cells was previously shown to spread to neighboring cells (Hirose et al., 1999). We do not know the extent of the spread of this calcium wave in our preparations because the high magnification required for visualization of a cilium reduced the field of view to the cell under study and one additional cell diameter. Such calcium waves are generally mediated by IP_3 spreading from cell to cell through gap junctions (Boitano, Dirksen & Sanderson, 1992) or by ATP or UTP as an intercellular messenger (Osipchuk & Cahalan, 1992; Lazarowski & Boucher, 2001). In our study, both cilium bending and pressure on the apical membrane resulted in a calcium wave that spread through gap junctions, since an inhibitor of gap junction permeation, heptanol, significantly reduced the spread. This indicates that the calcium waves are transmitted by a similar mechanism in both types of experiments. The lack of complete inhibition by the PLC inhibitor, U73122, of the response to bending of the cilium by micropipette could indicate that other messengers, such as ATP or UTP, are also involved in the generation or propagation of the calcium transient. Involvement of apical ATP release as the mechanism of spread of the calcium signal was ruled out in preliminary experiments as the calcium wave generated by pressing the membrane was unaffected by the addition of the enzyme apyrase (*data not shown*). It was not possible to study the effects of U73122 on the lateral spread after bending the cilium by micropipette because of the markedly reduced calcium signal in the primary cell. However, it seems most likely that IP_3 is the promoter of the intercellular calcium waves, because U73122 completely blocks the lateral spread induced by pressing the apical membrane. Increased perfusate flow results in a widely spread increase in intracellular calcium that could be the result of a comparable response from all cells or spread of the response from a few "responsive" cells to their neighbors. We cannot discriminate between these possibilities at this time, but a determination is experimentally feasible.

The profound transient hyperpolarization that accompanies perfusate flow is probably only one consequence of the large increase in intracellular calcium that occurs. Many other calcium-sensitive processes should also be affected. Because of their transient nature, such phenomena may have been dismissed in previous studies of epithelial membrane potential or transport properties.

Since the primary cilium of MDCK cells responds to flow it seems highly likely that it serves a similar function in the native renal collecting duct. Renal tubular epithelial cells from the proximal tubule to the collecting duct all exhibit a primary cilium (Latta et al., 1961; Pfaller & Klima, 1976). The flow sensitivity of the primary cilium could constitute the long sought mechanism for "glomerular-tubular balance," a phenomenon in which the rate of fluid absorption by proximal tubule cells varies in proportion to the glomerular filtration rate (Guo, Weinstein & Weinbaum, 2000). The flow dependence of cortical collecting duct functions, such as potassium secretion and transepithelial potential (Engbretson & Stoner, 1987; Malnic, Berliner & Giebisch, 1989), has never been explained, although it has been speculated that the primary cilium in the collecting duct could be a flow sensor (Allen, 1965; Wiederhold, 1976; Wilsman, 1978; Schwartz et al., 1997). If bending the primary cilium results in a substantial increase in intracellular calcium of long duration as in MDCK cells, the calcium-activated K^+ channels previously identified in these cells (Breuer, Mack & Rothstein, 1988) would be expected to open and thus stimulate K^+ secretion.

A single primary cilium occurs in a vast number of nonrenal tissues and cell types (Wheatley, Wang & Strugnell, 1996), and its function as a mechano-sensor is possible in other tissues subject to flow such as endothelial cells, in which primary cilia have been detected (Edanaga, 1974; Ekblom & Hansson, 1984) and where the flow dependence of permeability has been attributed, until now, to shear forces (Waters, 1996). In addition, the primary cilium may act as a chemical sensor, sampling the apical perfusate or renal tubule lumen for an unknown, but critical, solute. Finally, the refractory period following bending the cilium is consistent with depletion of intracellular calcium stores, a result that may explain the "feeding effect" seen in cultured epithelial

cells, in which the properties of cultures may differ from baseline level for an extended period after changing the medium.

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