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Released nucleotides amplify the cilium-dependent, flow-induced $[Ca^{2+}]_i$ response in MDCK cells

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Abstract

Aim: Changes in perfusate flow produce increases in $[Ca^{2+}]_i$ in renal epithelial cells. Cultured renal epithelia require primary cilia to sense subtle changes in flow. In perfused kidney tubules this flow response is caused by nucleotide signalling via $P2Y_2$ receptors. It is, however, not known whether nucleotides are released by mechanical stress applied to renal primary cilia. Here we investigate whether nucleotides are released during the cilium-dependent flow response and contribute to the flow-induced, cilium-dependent $[Ca^{2+}]_i$ signal.

Methods: MDCK cells loaded with Fluo-4-AM were observed at 37 °C in semi-open single or closed-double perfusion chambers.

Results: Our data suggest a purinergic component of the cilium-dependent flow-response: (1) ATP scavengers and P2 receptor antagonists reduced (55%) the cilium-dependent flow-response; (2) ATP added at subthreshold concentration sensitized the renal epithelia to flow changes; (3) increases in fluid flow transiently enhanced the ATP concentration in the superfusate (measured by biosensor-cells). To test if nucleotides were released in sufficient quantities to stimulate renal epithelia we used non-confluent MDCK cells without cilia as reporter cells. We confirmed that non-confluent cells do not respond to changes in fluid flow. Placing confluent, ciliated cells upstream in the in-flow path of the non-confluent cells made them responsive to fluid flow changes. This phenomenon was not observed if either non-confluent or de-ciliated confluent cells were placed upstream. The $[Ca^{2+}]_i$-response in the non-confluent cells with ciliated cells upstream was abolished by apyrase and suramin.

Conclusion: This suggests that subtle flow changes sensed by the primary cilium induces nucleotide release, which amplifies the epithelial $[Ca^{2+}]_i$-response.

Keywords ATP, Ca^{2+}, cilia, MDCK pressure, mechano-sensation, P2Y.

Renal tubular flow of pre-urine shows large variations and renal epithelial cells are potentially subjected to significant changes in shear stress. As primary cilia protrude from the centriole into the lumen of the kidney tubules, its position and bending properties led to speculations that it might be a sensor of tubular flow (Schwartz *et al.* 1997). This hypothesis has been substantiated in a number of studies in cultured renal epithelia (Praetorius & Spring 2001, 2003, Nauli *et al.* 2003, Xu *et al.* 2006, Kotsis *et al.* 2007). In renal cell culture primary cilia render the cells more sensitive for subtle changes in fluid flow rates (Praetorius & Spring 2001, 2003), which favours that this organelle is indeed functioning as a mechano-sensory structure. Studies of perfused rabbit collecting duct indicate that an increase in perfusate flow also stimulates $[Ca^{2+}]_i$ elevations in the native tissue (Woda *et al.* 2002). This $[Ca^{2+}]_i$ elevation, however, occurs both in principal and intercalated cells,

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where the latter do not express primary cilia (Andrews & Porter 1974). This suggests either that primary cilia are not a prerequisite for flow sensing in perfused tubules or that the tissue has a system for intercellular communication. In this context, one should note that the epithelial cells are not stimulated in the same way in a perfusion flow chamber and perfused tubules. In the latter case, the epithelium is removed from the tissue and has lost its lateral support. Changes in perfusion flow rates will therefore result in both an increase in apical laminar flow and an increase in tubular diameter (Woda *et al.* 2001, Jensen *et al.* 2007) inflicting lateral stretching of the epithelium. Increases in the transepithelial pressure produce transient $[Ca^{2+}]_i$ increases in MDCK cells via nucleotide release and autocrine and paracrine stimulation of P2 receptors (Praetorius *et al.* 2004); a response independent of primary cilia. These findings are supported by studies of freshly isolated perfused tubules (Jensen *et al.* 2007). In the medullary thick ascending limb from mice, acute changes in flow rates result in prompt $[Ca^{2+}]_i$ increases that requires purinergic signalling as the response is abolished by ATP scavengers, P2 receptor antagonists and significantly blunted in $P2Y_2$ receptor-deficient mice (Jensen *et al.* 2007). Because of the aforementioned limitations, the results are not conclusive as to whether primary cilia are involved in the flow-induced response in isolated perfused renal tubules. The current project re-investigated the flow-stimulated $[Ca^{2+}]_i$ increase in the MDCK cell model and addresses if stimulated nucleotide release occurs secondary to bending the primary cilium.

Methods

Cell culture

Wild-type MDCK type 1 cells (passages 54–70 from the American Type Culture Collection, Rockville, MD, USA) were grown to confluence on 25 mm diameter coverslips or permeable supports (Anopore, 0.2 μm; Nunc/VWR-International, Albertslund, Denmark) in Dulbecco's modified Eagle medium with 10% foetal bovine serum (Gibco, Invitrogen, Taastrup, Denmark) 2 mM glutamine, 1 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, but without riboflavin as described previously (Praetorius & Spring 2001, 2003).

Microscopy and perfusion

MDCK cell mono-layers, on coverslips or Anopore filters, were viewed at 37 °C on the stage of an inverted microscope (TE-2000; Nikon) equipped with differential interference contrast combined with low light level fluorescence provided via a Xenon lamp and monochromator (Visitech International, Sunderland, UK). Imaging was performed with a long distance plan Fluo 20X, 0.45 NA or a 100X/1.4 NA. Plan Apo lens (Nikon), an intensified SVGA CCD camera and imaging software (Quanticell 2000/Image Pro; VisiTech International). The cellular fluorescence was sampled at the rate of 0.5 Hz and measurements were initiated 40 s prior to start of perfusion.

We used two types of perfusion chambers: a semi-open chamber and a closed chamber with perfusion of both the apical and basal side of the cultured cells. The semi-open chamber is the commercially available Warner Instruments chamber: RC-21BRFS, modified to be semi-open, which means that the perfusion chamber is covered by only half a coverslip (Warner Instruments, Hamden, CT, USA). In this way one avoids the building up of pressure in the system, reduce evaporation compared to completely open chambers and still keep the good optical properties of a closed chamber. Solutions were perfused at constant flow rates of 12 μL s^{-1}, which corresponds to a bulk flow velocity of 800 μm s^{-1}.

The custom-made double-sided cell chamber is a closed perfusion system, which in contrast to the semi-open chamber allows the build up of pressure gradients over the cell layer. The cell chamber consists of two symmetrical compartments separated by the Anopore filer upon which the MDCK cells were grown (Praetorius *et al.* 2004). The inner dimensions of the two compartments in this slit shaped chamber were 6 (length) x 1 (width) x 2 (height) mm. Solutions were perfused at constant flow rates of 1.7 μL s^{-1}, which corresponds to a bulk flow velocity of 800 μm s^{-1}. To build up a pressure gradient over the epithelium (short pressure pulses) it was necessary to discontinue the flow on the particular side to which the pressure was to be applied by blocking the out-flow line. The pressure pulse was induced by rapid compression of the inflow line with a metal clamp and resulted in transient transepithelial pressure pulses of 80 mmHg (Praetorius *et al.* 2004). The opposite compartment was continuously perfused unless stated otherwise.

Intracellular Ca^{2+} measurements by Fluo-4

The cells were incubated for 30 min with the Ca^{2+}-sensitive probe Fluo-4-AM (5 μM) at 37 °C, and washed twice to remove excess probe. They were then placed in the perfusion chamber and allowed at least a 20-min de-esterification period. Fluo-4 was excited at 488 nm and emission was detected above 520 nm. The fluorescence intensity was expressed relative to the baseline value.

Solutions

The perfusion solution had the following composition, in mM: $[Na^+]$ 138, $[K^+]$ 5.3, $[Ca^{2+}]$ 1.8, $[Mg^{2+}]$ 0.8,

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[Cl^-] 126.9, [SO_4^{2-}] 0.8, Hepes 14, glucose 5.6, probenecid 5, pH 7.4 (37 °C, 300 mOsmol L^{-1}). The Ca^{2+}-free solution had the following composition, in mM: [Na^+] 139, [K^+] 5.3, [Mg^{2+}] 0.8, [Cl^-] 125.3, [SO_4^{2-}] 0.8, EGTA 1, Hepes 14, glucose 5.6, probenecid 5, pH 7.4 (37 °C, 300 mOsmol). Sources of chemicals were: Fluo-4-AM (Invitrogen), EGTA, probenecid, apyrase (grade 1) and suramin (Sigma, St Louis, MO, USA). All solutions contained 5 mM probenecid to inhibit extrusion of the dye, and the experiments were carried out at 37 °C, pH 7.4.

Statistics

All values are shown as the mean ± SEM. Statistical significance was determined using the Mann–Whitney–Wilcoxon nonparametric test for comparison of two groups and the one-way ANOVA followed by a Tukey–Kramer multiple comparison test for comparison of more than two groups. In both cases a *P*-value less than 0.05 was considered significant. In each experiment (preparation) ~30 cells were chosen randomly at the first picture in the image sequence during the baseline period and before the response pattern of the given cells were known. *n* refers to the number of coverslips/filters tested.

Results

ATP scavenging reduces the flow response

In a double perfusion chamber, where the two chambers are separated only by cells grown on a permeable support, we had access to both the apical and basolateral side of the renal epithelial cells in culture. In this type of chamber, we were able to add the ATP scavengers or P2 receptor antagonists to both sides of the epithelium. Figure 1 shows how bilateral addition of the ATP scavenger apyrase or the P2 receptor antagonist suramin reduced the flow response.

Our experiments revealed that the flow-induced response in the closed, double perfusion chamber differed significantly from our known cilium-dependent one in the semi-open chamber. It occurred more

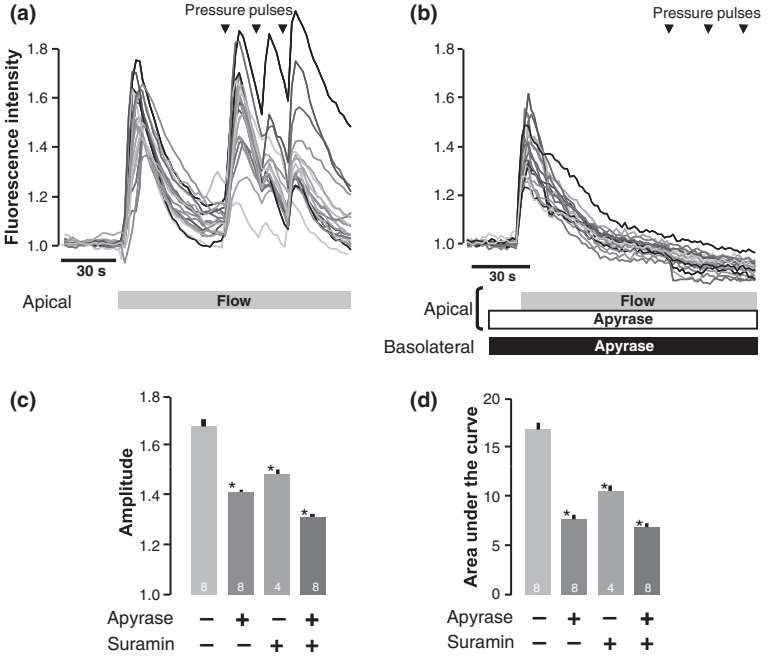

Figure 1 (a) The flow-induced $[Ca^{2+}]_i$ increase in a closed double-perfusion chamber in MDCK cells grown to confluency on permeable filters. The figure shows representative traces of increase in relative Fluo-4 fluorescence from 19 cells to a change in apical flow rate of 0–2 μL s^{-1}. The basolateral side was not perfused during the experiments. Immediately after the trace returns to baseline levels the cells are re-stimulated with trans-epithelial pressure pulses of a magnitude of approx. 80 mmHg (n = 8). (b) The effect of bilateral apyrase on the flow-induced $[Ca^{2+}]_i$-response in confluent MDCK cells. Confluent MDCK cells were exposed to the same protocol as shown in (a) in the presence of 5 U mL^{-1} apyrase on both sides of the epithelium (n = 8). (c) The summarized results of the amplitude of the $[Ca^{2+}]_i$ -response in the control situation and in the presence of bilateral apyrase (5 U mL^{-1}) and/or suramin (1 mM). The effect of suramin alone was evaluated on the basis of four preparations, and the combined treatment of apyrase and suramin is the average of eight preparations. (d) The summarized results for the area under the curve of the $[Ca^{2+}]_i$-response in the control situation and in the presence of bilateral apyrase (5 U mL^{-1}) and/or suramin (1 mM).

abruptly and synchronized (compare with Fig. 3) and did not show the well described refractoriness to re-stimulation (Fig. S1). We decided to test the flow response in the closed perfusion chamber for some of the characteristics of the cilium-dependent flow response. The cilium-dependent flow response is known to require apical Ca^{2+} (Praetorius & Spring 2001, 2003). In the double perfusion chamber it was apparent that a flow-induced response could still be provoked in the absence of extracellular Ca^{2+} even though it was considerably smaller. In addition, the flow response was also visible in non-confluent cells that do not express primary cilia (Fig. S1). We believe that these discrepancies between the cellular response to flow changes in closed versus open perfusion systems reflect a difference in mechanical perturbation as flow is initiated. In the closed perfusion systems (perfusion chamber or perfused tubule) the mechanical stimulation is a compound effect of the increase in luminal flow and stretch of the epithelial layer. Consequently, the double chamber is not suitable to investigate the cilium dependency of the ATP release. Therefore the following experiments were performed in the semi-open chamber where the flow response is known to be cilium dependent. Nonetheless, Figure 1 clearly supports that nucleotide release is a consequence of flow stimulation.

ATP sensitizes the cilium-dependent flow response

At first, the concentration–response relationship of ATP as a $[Ca^{2+}]_i$ agonist was measured in MDCK cells (Fig. 2a). Concentrations above 0.4 μM ATP induced significant $[Ca^{2+}]_i$ increases (EC_{50} 1.65 μM). The flow–response curve was then investigated in the presence of subthreshold concentrations of ATP (0.1 and 0.3 μM). Figure 2b illustrates that with subthreshold concentrations of ATP the MDCK cells become much more susceptible to fluid flow changes. The presence of extracellular ATP also reduced the time from stimulus to maximal $[Ca^{2+}]_i$ response (data not shown). Together, these data indicate that flow sensitivity is dependent on purinergic receptor input, likely via apical $P2Y_2$ receptors. The ATP-induced leftward shift of the flow response curve is in agreement with the notion that flow triggers nucleotide release from MDCK cells.

Flow-stimulated nucleotide release

We tested whether scavenging of apical ATP affected the cilium-dependent flow response (Fig. 3). Apyrase (10 U mL^{-1}) led to a ~50% reduction of the amplitude of the flow-induced $[Ca^{2+}]_i$-response. Therefore, these results support that a flow stimulus triggers the release of nucleotides and paracrine activation of luminal P2 receptors.

We tried various approaches to substantiate whether the renal epithelial cells release nucleotides as a consequence of cilium bending. The methods for measuring ATP release are not sensitive enough to register ATP release from a single cell after direct manipulation of a mono-cilium. Therefore, we tried to establish whether ATP was released from a sheet of epithelial cells during application of flow in a system where the flow response was established to be cilium dependent. We took advantage of 132-1N1 astrocytoma cells transfected with the human $P2Y_2$ receptor as biosensor. These cells are highly sensitive to ATP (10^{-8} M; Fig. 4c), whereas the wild type cells are non-responsive as they do not express P2 receptors. By collecting the superfusate from MDCK cells at various intervals (before flow, 0–1 and 1–2 min after flow start) and subject the biosensors to the superfusate we were able to detect ATP in the collected perfusion solution. The superfusate from MCDK cells was collected from 0–1 (720 μL) and 1–2 min (720 μL) after the flow was started and immediately boiled and frozen. These superfusate sam-

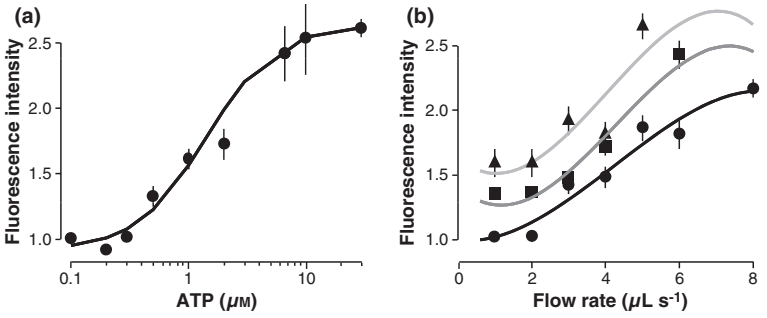

Figure 2 The effect of subthreshold ATP on the flow-induced $[Ca^{2+}]_i$-response in MDCK cells. (a) The graph shows the experimental concentration–response relationship of the ATP-induced $[Ca^{2+}]_i$-increase. Twelve preparations were tested all together. (b) The effect of extracellular ATP on the flow-induced $[Ca^{2+}]_i$-response. The circles represent the control flow–response curve of untreated MDCK cells. The squares represent the same curve with 0.1 μM ATP in the apical fluid. The triangles represent the same situation with 0.3 μM in the apical fluid at all times. The total number of preparations tested was 21.

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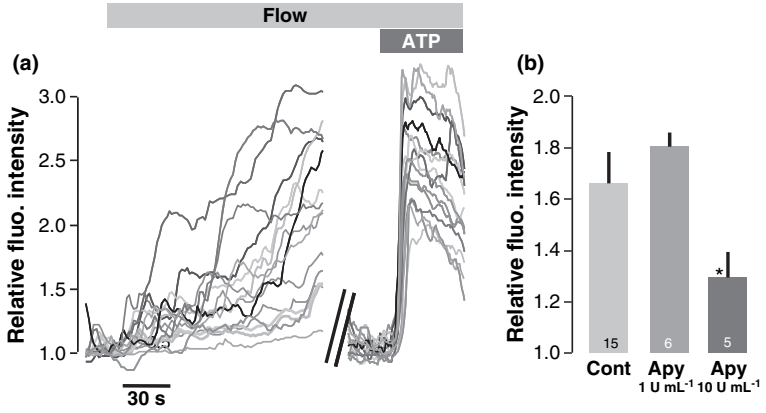

Figure 3 (a) The flow-induced $[Ca^{2+}]_i$-increase in a semi-open perfusion chamber for cells grown on coverslips. The traces on the left show the relative Fluo-4 fluorescence increase in 13 cells as a result of change in superfusate flow rate from 0 to 12 μL s^{-1} in a single experiment. Immediately after the traces returns to baseline levels (after approx. 3 min) the cells are re-stimulated with 100 μM ATP (n = 17). (b) Effect of apically applied apyrase. The summarized data of flow-induced $[Ca^{2+}]_i$ increase (0–12 μL s^{-1}) in the absence or presence of apyrase (1 and 10 U mL^{-1}). The number in the columns refers to the number of preparations tested.

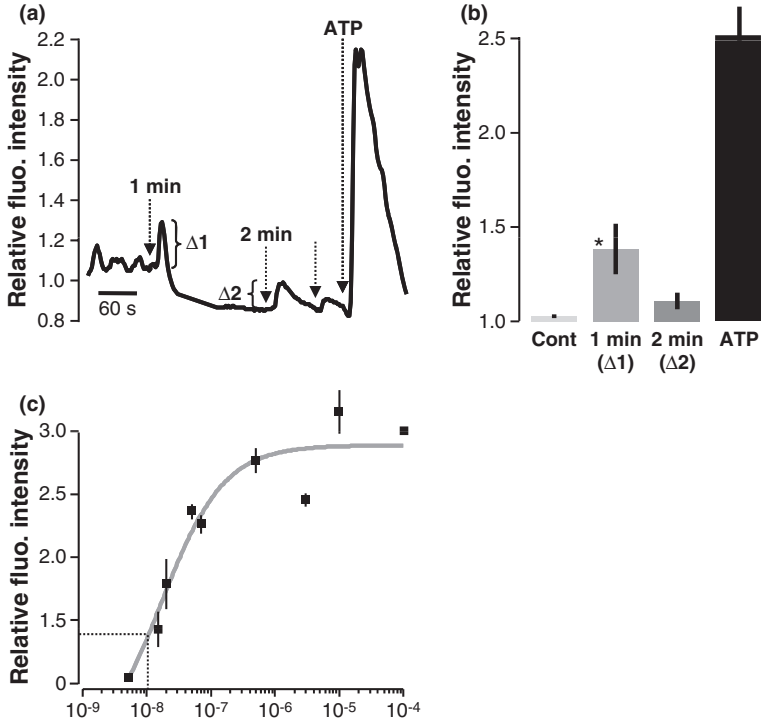

Figure 4 Changes in Fluo-4 fluorescence in 132-1N1 astrocytoma cells transfected with the hP2Y$_2$ receptor. (a) The 132-1N1 cells were used as biosensors for ATP in MDCK cell superfusate. The superfusate collected from MDCK cells before and in the interval 0–1 and 1–2 min after the flow was initiated was boiled immediately after collection. Afterwards, samples of this superfusate were added to bio-sensor cells as indicated by the arrow and the amplitude of the signal was measured. ATP (100 μM) was added as a control for the responsiveness of the cells. (b) Summarized data. (c) Concentration–response curve for the biosensor cells. The average amplitude measured by adding the supernatant collected over 0–1 min from the MDCK cells is indicated by the horizontal line.

ples resulted in small, transient increases in $[Ca^{2+}]_i$ when added to the medium covering the biosensor cells (Fig. 4a). ATP (100 μM) was used to assure that the biosensor cells were responding in a regular fashion to known concentrations of ATP. Figure 4b summarizes the data from four experiments. Using this concentra-

tion–response for the ATP curve (Fig. 4c) as reference, the perfusate-induced changes in $[Ca^{2+}]_i$ to the biosensor cells allows crude quantification of the ATP concentration in the sample. Taking the dilution factor into account the superfusate nucleotide concentration is ~60 nM. Addition of MDCK cell superfusate did not provoke $[Ca^{2+}]_i$ changes in wild type 132-1N1 cells. These did, however, react normally to carbachol (data not shown).

To evaluate the relevance of the flow-induced nucleotide release, it is necessary to know, if ATP is released in concentrations sufficient to stimulate the MDCK cells themselves. To this end we used MDCK cells as nucleotide reporter cells. We took advantage of the inability of non-ciliated, non-confluent MDCK cells to react to flow changes (Praetorius & Spring 2001, 2003). As they are not activated by changes in fluid flow rate, they can report about potential paracrine factors released during flow stimulation of confluent, ciliated MDCK cells. This principle of paracrine factors washing over from one sheet of cells to another was introduced by Charles (1998). We used cells grown on coverslips cut in half. In this way, we could place cells of different states of confluency in the same flow chamber. Figure 5 (and Movie S1) shows the control condition, where non-confluent cells on half coverslips were placed next to each other separated only by a small gap (~40 μm). When the flow was started practically no rise in $[Ca^{2+}]_i$ was observed in the non-confluent cells on either side. The cells were however very responsive to ATP. The lack of cilia in non-confluent cells was verified with inspection at high magnification and by immunocytochemistry as described previously (Praetorius & Spring 2001, 2003).

We then performed a similar experiment with confluent, ciliated cells placed upstream in the flow path. The ciliated cells responded to increases in fluid flow rates by a robust increase in $[Ca^{2+}]_i$ as reported previously (Praetorius & Spring 2001) (Fig. 6a, Movie S2). Under these conditions the previously unresponsive, non-ciliated cells displayed a significant increase in $[Ca^{2+}]_i$ when flow was initiated (Fig. 6b). This finding is consistent with a stimulating factor being washed from the confluent cells onto the non-confluent cells. To investigate whether this phenomenon involves nucleotides we tested whether apyrase or suramin might alter this signal. Apyrase (10 U mL^{-1}) and suramin (1 mM) completely abolished the $[Ca^{2+}]_i$ response in the non-confluent cells after elevation of the superfusate flow rate (Fig. 7). These results strongly indicate that the washable factor is a nucleotide.

The question remains as to whether ATP is released by the mechanical stimulation or whether the fluid flow mixes up an otherwise unstirred fluid layer close to the apical cell surface, which contains sufficient extracellular ATP to stimulate the reporter cells. If the

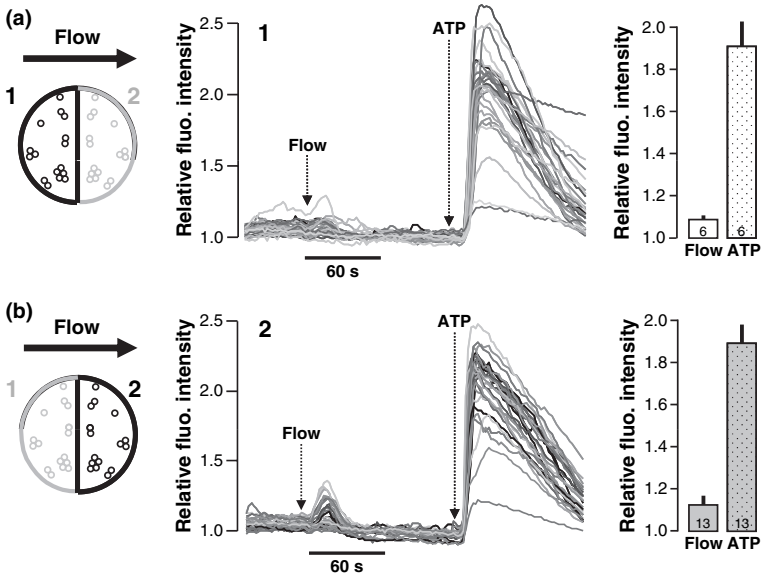

Figure 5 Non-confluent MDCK cells without primary cilia do not respond to increases in fluid flow rates. Non-confluent MDCK cells, which do not express primary cilia, were grown on half coverslips and inserted in the semi-open perfusion chamber. (a) $[Ca^{2+}]_i$ changes in response to increase in superfusion flow rate (12 μL s^{-1}) and ATP (100 μM) in the non-confluent cells upstream in the perfusion chamber. Representative traces from a single experiment are shown to the left and the summarized data from all preparations tested to the right. (b) $[Ca^{2+}]_i$ changes in response to flow (12 μL s^{-1}) and ATP (100 μM) in the non-confluent MDCK cells downstream from the partition between the cells. Representative traces from a single experiment are shown to the left and the summarized data from all preparations tested to the right.

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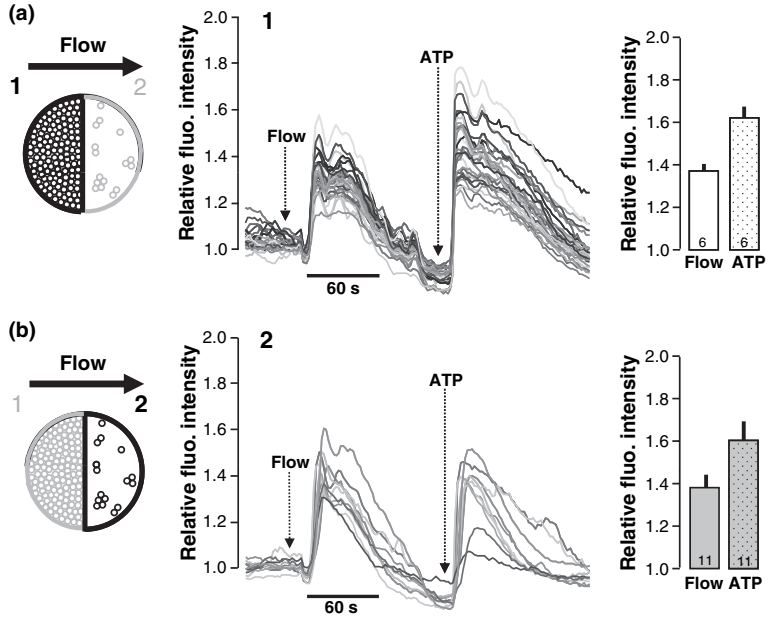

Figure 6 Release of a paracrine factor during the cilium-dependent flow–response. (a) $[Ca^{2+}]_i$ changes in response to increase in superfusion flow rate (12 μL s^{-1}) and ATP (100 μM) in the confluent MDCK cells upstream in the perfusion chamber. Representative traces from a single experiment are shown to the left and the summarized data from all preparations tested to the right. (b) $[Ca^{2+}]_i$ changes in response to flow (12 μL s^{-1}) and ATP (100 μM) in the non-confluent MDCK cells downstream from the partition between the cells. Representative traces from a single experiment are shown to the left and the summarized data from all preparations tested to the right.

latter was the case our data could simply reflect the higher number of cells when confluent cells are placed upstream from the reporter cells compared with non-confluent cells. We tried to investigate this further by a preparation of confluent cells lacking primary cilia. MDCK cells were treated for 4 days with 4 mM chloral hydrate, known to de-ciliate MDCK cells by destabilizing the microtubules (Praetorius & Spring 2003). After the removal of the primary cilia the cells were allowed to recover for 24–48 h to normalize the microtubular pattern. We previously have shown that the flow response in MDCK cells only returns after the primary cilia have fully re-grown on day 4 after chloral hydrate treatment. Figure 8 shows the $[Ca^{2+}]_i$ response in non-confluent cells with either control confluent cells upstream (Fig.8a) or confluent, de-ciliated cells upstream (Fig. 8b). With the non-ciliated, confluent cells in the flow path the washable effect on $[Ca^{2+}]_i$ in the reporter cells was completely absent. These results indicate that the presence of primary cilia is required for flow-stimulated nucleotide release.

Discussion

MDCK cells originate from connecting tubules and collecting ducts of the canine kidney and are able to respond to changes in apical flow rate with a $[Ca^{2+}]_i$ transient (Praetorius & Spring 2001, 2003). The cells depend on primary cilia to sense subtle changes in superfusate flow rate (Praetorius & Spring 2001, 2003). Mechanical stress applied to the cilium induces an intracellular Ca^{2+} response that initially depends on Ca^{2+} entry, but also involves release from IP_3-sensitive Ca^{2+} stores (Praetorius & Spring 2001, 2003). Mechanical perturbations of cells very often provoke release of intracellular nucleotides as local signalling molecules. We have recently shown that increases in fluid flow rates in perfused renal tubules ignite $[Ca^{2+}]_i$ responses via nucleotides as paracrine messengers (Jensen *et al.* 2007). These flow-induced $[Ca^{2+}]_i$ responses have some similarities to the cilium-dependent flow response, but also show marked differences. In the perfused tubule there is no absolute requirement for initial apical Ca^{2+} influx, and the response does not show the same temporal delay of onset as described for the cilium-dependent flow response (Praetorius & Spring 2001, 2003, Jensen *et al.* 2007). One likely explanation is that the mechanical stimulation of the cells is quite different in the two set-ups. The cilium-dependent flow response was measured in a semi-open chamber, where subtle changes in fluid flow rates produce minimal pressure increases in the chamber. In the perfused tubule raising the inflow pressure produces an increase in laminar flow, and increase in pressure and thus a lateral

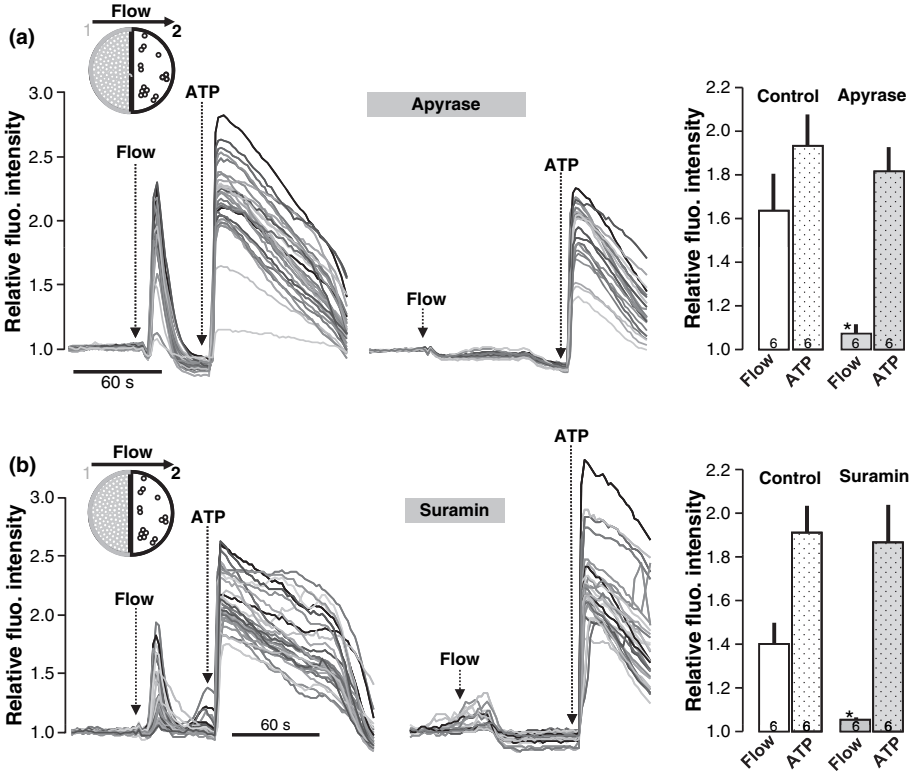

Figure 7 The effect of apyrase and suramin on the cilium-dependent flow response. (a) An original trace of non-confluent cells without cilia, with confluent, ciliated cells placed upstream. The trace to the left shows the control and the trace to the right shows the response in the presence of apyrase (10 U mL^{-1}). The bar graph to the right sums up all experiments. (b) Original traces from a single experiment of non-confluent cells without cilia, with confluent, ciliated cells placed upstream. The trace to the left shows the control and the trace to the right shows the response in the presence of suramin (1 mM). The bar graph to the right sums up data from all preparations.

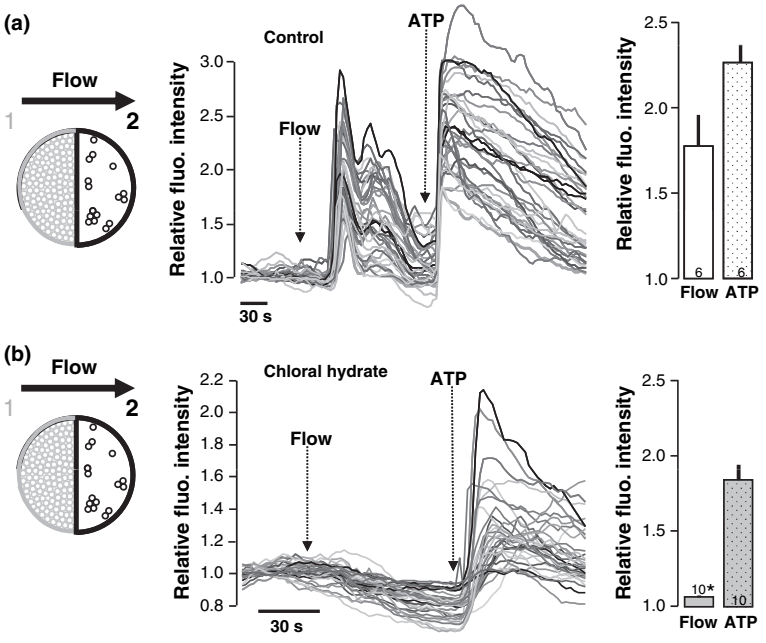

Figure 8 The effect of de-ciliating the confluent MDCK cells with chloral hydrate on the flow-induced nucleotide release. (a) $[Ca^{2+}]_i$ increase in the non-confluent cells in response to flow increase with confluent ciliated MDCK cells upstream, and (b) with confluent cells devoid of primary cilia upstream. The bar graph to the right sums up data from all preparations.

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distension of the epithelium (Woda *et al.* 2001, 2002, Jensen *et al.* 2007). This resembles what one can detect in closed perfusion chambers. When cells are observed at high magnification, there is a focus shift as the inflow pressure is raised. Refocusing revealed that the coverslip was bent towards the objective lens. This is likely to create a mechanical stress to the epithelial cells, which does not occur in an open chamber, where flow changes do not provoke focus shifts (data not shown). This mechanical distension is especially obvious in our customized double perfusion chamber, where the two chambers are only separated by the epithelial cells grown on filters. In this system compression of the inflow lines can produce pressure gradients over the epithelium, which triggers nucleotide release (Jensen *et al.* 2007). In this study, we performed flow experiments in the closed perfusion chamber and found that the characteristics of the flow response to be similar to that observed in the perfused tubules. The flow response loses its absolute dependency on extracellular Ca^{2+}. In this context, it is noteworthy that the flow response in the closed chamber no longer has an absolute requirement for primary cilia. These findings strongly indicate that if the perfusate flow rates in closed systems are high enough to produce additional mechanical stimulation the primary cilium dependency of the flow-induced signal is lost. This implicates that epithelial cells are likely to release nucleotides when the stimulus is large enough and that primary cilia renders the cells more susceptible for subtle mechanical changes such as flow. This is supported by recent data from bile duct epithelia and endothelial cells where the flow response is sensitized in the presence of primary cilia (Hierck *et al.* 2008, Woo *et al.* 2008). In intact renal tubules it is still not settled whether primary cilia sensitize the epithelial cells to react to flow. There are, however, interesting data showing that mice with shortened primary cilia ($tg737^{-/-}$) display a reduced flow response in the intact collecting duct (Liu *et al.* 2005). These data are further supported by a study of perfused bile ducts where the flow response was reduced after cilia removal with chloral hydrate (Masyuk *et al.* 2006).

In the light of what is discussed above, it is still not clear whether nucleotides are released during the cilium-dependent flow response. Recent results indicate that cultured renal epithelial cells with defective cilia assembly have impaired spontaneous and flow stimulated nucleotide release (Hovater *et al.* 2008). In the present study, we were able to show that externally applied nucleotides (in concentrations too low to induce $[Ca^{2+}]_i$ increases by themselves) sensitized the cilium-dependent flow response. Furthermore, we showed that scavenging apical ATP reduces the amplitude of the flow-induced $[Ca^{2+}]_i$ response, and that the amount of ATP in the superfusate does increase as the fluid flow rate is enhanced.

These findings were substantiated by a method to detect the release of paracrine factors during the cilium-dependent flow response. We used non-confluent, non-ciliated MDCK cells unable to respond to fluid flow changes as reporters for paracrine factors washing from responding ciliated cells. We confirmed that the non-ciliated, non-confluent cells did not respond to flow changes, but were highly responsive to ATP. When confluent ciliated cells were placed upstream in the flow chamber the non-confluent cells became responsive to fluid flow changes. This signifies that a stimulatory factor was washed from the sensing cells to the reporter cells. These paracrine factors are by all likelihood nucleotides, as the ATP scavenger apyrase and the non-selective P2 receptor blocker suramin abolished the response in the non-confluent cells. We cannot exclude that more signalling molecules may play a role in this effect. We feel safe to conclude that one is likely to be ATP as removal of this signal substantially abrogates the flow-induced response in the reporter cells.

The question was whether nucleotides are actually being released by the mechanical stimulation or whether flow just stirred up an ATP aura covering the resting cells. If this was the case the responsiveness of the non-confluent reporter cells would be a function of the number of cells in the inflow path. This did not seem to be the case as non-confluent cells remained irresponsive when confluent, de-ciliated cells where placed upstream. These findings make us conclude that a paracrine factor is released during the cilium-dependent flow response and that this paracrine factor is ATP. With our current technique we were not able to detect whether UTP is released during the cilium-dependent flow response. In this context it is worth noticing that the Ca^{2+} influx channel required for the cilium-dependent flow response recently has been shown to be $TRPV_4$ forming a complex with TRPP2 (Kottgen *et al.* 2008). This is of particular interest in our context as the $TRPV_4$ channel in addition to be mechanically activated has been shown to facilitate ATP release in the urinary bladder (Gevaert *et al.* 2007) and the medullary thick ascending limb (Silva & Garvin 2008).

With regard to the physiological relevance of these findings, renal epithelial cells *in situ* will experience changes in fluid flow as a function of varying urinary flow rates. In addition to steady changes in laminar flow a large part of the renal tubular system experiences oscillatory changes in fluid flow and pressure, i.e. both perfused and non-perfused proximal tubules (Holstein-Rathlou 1987) and in the distal tubules (Schmidt-Nielsen & Graves 1982). The latter are induced by papillary contractions between which the lumen of the tubules distends as boluses of urine pass the tubule

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(Schmidt-Nielsen & Graves 1982). These distensions ought to result in stretching of the epithelia that resemble what is seen in the isolated perfused tubule. Taken together, the types of mechanical perturbations described here (ciliary sensing of subtle changes in fluid flow rates and abrupt stretching of the renal epithelium) might both turn out to be relevant for the normal kidney function. In this regard, it is worth noticing that mice deficient in the major P2 receptor in the distal renal tubules, the $P2Y_2$ receptor, have a significant renal phenotype: in addition to increased blood pressure, it has distal tubular hyper-absorption and, thus, a reduced ability to dilute its urine (Rieg *et al.* 2007). Our present finding supports that mechanically induced nucleotide release is relevant for integrated renal tubular function. Our data suggest that the nucleotide signalling system is able to amplify and coordinate tissue responses to subtle stimuli.

Conflict of interest

The authors do not have any conflict of interest for this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Flow induced [Ca^{2+}]; response in MDCK cells in closed perfusion chamber.

Movie S1. Non-confluent reporter cells with non-confluent, non-ciliated cells upstream. The cells are subjected to increase in fluid flow followed by addition of ATP.

Movie S2. Non-confluent reporter cells with confluent, ciliated cells upstream. The cells are subjected to increase in fluid flow followed by addition of ATP.

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