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Bending the Primary Cilium Opens Ca^{2+}-sensitive Intermediate-Conductance K^+ Channels in MDCK Cells

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Abstract. Increasing tubular fluid flow rate has previously been shown to induce K^+ secretion in mammalian cortical collecting duct. The mechanism responsible was examined in the present study using MDCK cells as a model. The change in membrane potential difference (E_M) of MDCK cells was measured with a fluorescent voltage-sensitive dye, Di-$BAC_4(3)$, when the cell's primary cilium was continuously bent with a micropipette or by the flow of perfusate. Bending the cilium produced a hyperpolarization of the membrane that lagged behind the increase in intracellular Ca^{2+} concentration by an average of 36 seconds. Gd^{3+}, an inhibitor of the flow-induced Ca^{2+} increase, prevented the hyperpolarization. Blocking K^+ channels with Ba^{2+} reduced the flow-induced hyperpolarization, implying that it resulted from activation of Ca^{2+}-sensitive K^+ channels. Further studies demonstrated that the hyperpolarization was diminished by the blocker of Ca^{2+}-activated K^+ channels, charybdotoxin, whereas iberiotoxin or apamin had no effect, results consistent with the activation of intermediate-conductance Ca^{2+}-sensitive K^+ channels. RT-PCR analysis and sequencing confirmed the presence of intermediate-conductance K^+ channels in MDCK cells. We conclude that the increase in intracellular Ca^{2+} associated with bending of the primary cilium is the cause of the hyperpolarization and increased K^+ conductance in MDCK cells.

Key words: $DiBAC_4(3)$ — Ca^{2+} — Fluo-4 — Flow — Ba^{2+} — Charybdotoxin

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Introduction

The primary cilium, a solitary non-motile structure arising from the centriole and extending into the apical bathing solution, enables Madin Darby Canine Kidney (MDCK) cells to sense fluid flow (Praetorius & Spring, 2001). The intracellular Ca^{2+} concentration begins to increase a few seconds after the primary cilium is bent by manipulation with a micropipette or by the initiation of fluid perfusion. The maximal increase in intracellular Ca^{2+} concentration is achieved about 40 seconds after bending is initiated, and nearly five minutes are required for the Ca^{2+} concentration to return to baseline level. The increase in intracellular Ca^{2+} concentration caused by flow is accompanied by a large hyperpolarization of the membrane toward the calculated equilibrium membrane potential for K^+ (E_K) (Praetorius & Spring, 2001). Since MDCK cells are known to have Ca^{2+}-dependent K^+ channels (Breuer, Mack & Rothstein, 1988), this hyperpolarization might be due to activation of these channels.

In our previous study (Praetorius & Spring, 2001), MDCK cell E_M was measured with conventional glass microelectrodes. Several technical difficulties arose that severely limited the number of successful experiments. We showed that merely contacting the apical membrane of MDCK cells with a micropipette or microelectrode caused a transient increase in intracellular Ca^{2+} that lasted for about a minute. Thus, microelectrode punctures were required to remain stable for approximately two minutes before the experimental increase in perfusion rate could be performed. Such a response to mechanical contact is not unique to MDCK cells since nasal epithelial cells were reported to respond to mechanical stress with a transient increase in intra-

cellular Ca^{2+} of similar duration and amplitude and as a result of the release of ATP and UTP across both the apical and basolateral cell membranes (Homolya, Steinberg & Boucher, 2000). Frequently, increases in fluid flow rate caused dislodging or small displacements of the microelectrode that resulted in leaks or changes in the membrane potential (E_m). Finally, we could not manipulate a cell's primary cilium with a micropipette and simultaneously monitor its E_M with a microelectrode. Therefore, we employed an approach for E_M measurement in MDCK cells using a voltage-sensitive fluorescent dye, bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$).

In renal distal tubule, an increase in luminal flow rate is known to induce K^+ secretion and hyperpolarization of the transepithelial potential difference (Malnic, Berliner & Giebisch, 1989). K^+ secretion is flow-dependent for rates up to 6 nl/min in aldosterone-treated rabbit cortical collecting duct, above which it saturates (Engbretson & Stoner, 1987). In the rat distal tubule, flow-dependent K^+ secretion is reported to saturate between 20–30 nl/min (Malnic, Berliner & Giebisch, 1989). It has recently been suggested that this K^+ secretion in rabbit cortical collecting ducts is mediated through activation of Ca^{2+}-sensitive maxi K^+ channels (Tanigushi & Imai 1998; Woda et al., 2001; Woda et al., 2002). The exact nature of the K^+ channels and the signal transduction pathway for this response are, however, not well established.

It is our hypothesis that the intracellular Ca^{2+} concentration increase consequent to flow-induced bending of the primary cilium opens Ca^{2+}-sensitive K^+ channels in the apical membrane of MDCK cells, leading to a large hyperpolarization of E_M. Because of the similarities in the time course of flow-induced Ca^{2+} transients, transepithelial potential difference and K^+ permeability, we further hypothesize that a similar sequence of events occurs in renal tubules and is responsible for the flow dependence of K^+ secretion in the distal tubule and collecting duct.

Materials and Methods

Cell Culture

Wild-type 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 % fetal bovine serum (Gibco, Grand Island, NY) and 2 mM glutamine, but without riboflavin, antibiotics or phenol red, as previously described (Xia et al., 1984).

Solutions

The perfusion solution had the following composition, in mM: $[Na^+]$ 132, $[K^+]$ 5.3, $[Ca^{2+}]$ 1.8, $[Mg^{2+}]$ 0.8, $[Cl^-]$ 126.9, $[SO_4^{2-}]$ 0.8, HEPES 14, glucose 5.6, probenecid 5 in 3 ml NaOH, 1 N. Ba^{2+} solution contained, in mM: $[Na^+]$ 137, $[K^+]$ 5.3, $[Ca^{2+}]$ 1.8, $[Mg^{2+}]$ 0.8, $[Ba^{2+}]$ 5, $[Cl^-]$ 136.9, HEPES 14, glucose 5.6, probenecid 5. Low-chloride solution with Ba^{2+} contained, in mM: $[Na^+]$ 137, $[K^+]$ 5.3, $[Ca^{2+}]$ 8, $[Mg^{2+}]$ 0.8, $[Ba^{2+}]$ 5, $[Cl^-]$ 11.6, gluconate 131.3, HEPES 14, glucose 5.6, probenecid 5. All solutions were adjusted to pH 7.4 at 37°C and an osmolality of 300 mOsmol.

Microscopy and Perfusion

Continuously perfused MDCK cell monolayers were viewed 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).

The perfusion chamber had a 16 mm long, 3 mm deep and 3 mm wide flow path. 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^{-1} corresponds to a linear velocity of 11 μm sec^{-1}, equivalent to a tubular flow rate of 7 nl min^{-1}. The cellular fluorescence was sampled at a rate of 0.5 Hz during manipulation of a primary cilium. 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 approximated to the cilium tip, but was always at least 4 μM above the apical membrane. 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 50 seconds prior to the movement of the primary cilium. During these experiments, the cells were under constant slow perfusion of 2 μl sec^{-1}. In the flow experiments, the primary cilia of several cells were bent simultaneously by increasing the flow of perfusate to 8 μl sec^{-1}.

Intracellular Calcium Measured by Fluo-4

The cells were incubated for 15 minutes with the Ca^{2+}-sensitive probe Fluo-4-AM (5 μM) at 37°C, washed to remove excess probe and allowed at least a 20-minute de-esterification period. Then they were placed in the perfusion chamber and Fluo-4 fluorescence was measured as previously described (Praetorius & Spring, 2001). The fluorescence intensity was expressed relative to the baseline value, chosen as the mean of 5 intensity observations prior to experimental manipulation in a region of interest near the base of the cilium. All solutions contained 5 mM probenecid to inhibit extrusion of the dye, and the experiments were carried out at 37°C, pH 7.4.

Membrane Potential Measurements with $DiBAC_4(3)$

Changes in the membrane potential were measured with the fluorescent probe $DiBAC_4(3)$. The distribution of dye between the cell membrane and the cytosol is dependent on the membrane potential. Hyperpolarization results in more dye accumulation in the cell membranes, decreasing the signal arising from the cytosol and, therefore, the emitted fluorescence intensity of a region of interest within the cell (Bräuner, Hülser & Strasser, 1984). The relationship between the relative intensity of the fluorescence of a region of interest within the cell and the E_M was determined experimentally

in MDCK cells as described in Results. The cells were mounted in the perfusion chamber and incubated for 30 minutes with 1 μM $DiBAC_4(3)$. The dye remained in the perfusion solution throughout the duration of the experiment. The emitted fluorescence above 520 nm was measured at a sampling rate of 0.5 Hz using excitation at 488 nm. All perfusion solutions contained 5 mM probenecid to inhibit extrusion of the dye. Only ciliated cells were used for the measurements of E_M.

Reverse Transcription-Polymerase Chain Reaction and Sequence Analysis

Total RNA from wild-type MDCK cells was extracted using RNeasy Mini Kit (Qiagen, Germantown, MD) from confluent cells. After DNAse treatment, the RNA was reverse-transcribed by incubation with 2.5 U/μl Superscript Reverse Transcriptase (Life Technologies, Tåstrup, Denmark). Reverse transciptase was replaced by water in negative control samples. The cDNA was amplified by Polymerase Chain Reaction (PCR) using 50 U/ml Taq Polymerase (Boehringer-Mannheim, Mannheim, Germany). After 3 min at 95°C, 30 cycles of PCR amplification were performed with an annealing temperature of 60°C. The primers were derived from the published sequence for *Homo sapiens* and *Sus scrofa* intermediate-conductance K^+ channels (Genbank accession number AF022150 and AY062036, respectively). The primer sequences were: ICK channel, 5′ CCC CAT CAC ATT CCT GAC CA 3′ (sense) and 5′ GTG CAC GTG CTT CTC TGC CT 3′ (antisense), spanning 165 bp, corresponding to base 732 to 897 of the human sequence, AF022150; Actin (canine) 5′ GGC ACC AGG GTG TGA TGG T 3′ (sense) and 5′ GCG GTG GTG GTG AAG CTG TA 3′ (antisense), spanning 553 bp. The PCR products were analyzed by 2% agarose ethidium bromide gel electrophoresis and photography under ultraviolet illumination. Bands of predicted molecular weight for each PCR product were excised from the gel, purified by Gel extraction Kit (Qiagen) for sequence analysis (Lark Technologies, Essex, UK).

Materials

Sources of chemicals were: Fluo-4-AM, EGTA, probenecid, Gd^{3+} (Sigma, St. Louis, MO); r-Iberiotoxin, r-Charybdotoxin, and Apamin (Almone Labs, Jerusalem, Israel).

Statistics

All values are shown as the mean ± SEM. Statistical significance was determined using the nonparametric Mann–Whitney test; P values less than 0.05 were considered significant. The number of observations refers to the number of cells analyzed; typically, six cells were studied in each experiment.

Results

Measurements of E_M were conducted using a potential-sensitive fluorescent probe, $DiBAC_4(3)$, because previous experiments had shown that pressing the MDCK cell membrane during impalement by a micropipette produced a Ca^{2+} transient. Use of the dye avoided any unintended activation of the Ca^{2+} sensitive K^+ channels. The fluorescence of the dye was calibrated as described below to determine the relative change in E_M, although absolute values could not be obtained from the dye alone.

Flow-induced Ca^{2+} and E_M Changes

Increasing the apical perfusate flow rate from 2 μl sec^{-1} to 8 μl sec^{-1} produced an elevation in the intracellular Ca^{2+} concentration, as indicated by the increase in Fluo-4 fluorescence (Fig. 1*A*). The time course and amplitude of the Ca^{2+} increase were comparable to those reported previously with a peak increase occurring about 52 seconds after initiation of the increased flow rate (Praetorius & Spring, 2001). This Ca^{2+} response was associated with a significant hyperpolarization of E_M, as indicated by the 7.63 ± 0.32% (n = 150) decrease in the $DiBAC_4(3)$ fluorescence (Fig. 1*B*). As described in Methods, decreasing $DiBAC_4(3)$ fluorescence corresponds to hyperpolarization of the E_M. The maximal decrease was observed 88.0 ± 4.0 sec (n = 150) after the flow was initiated or about 36 seconds after the peak in Ca^{2+}. This time course is similar to that previously recorded with glass microelectrodes (Praetorius & Spring, 2001).

We previously showed that bending the primary cilium of a single cell with a micropipette results in an intracellular Ca^{2+} increase that is comparable in amplitude and time course to that produced by changing the apical flow rate (Praetorius & Spring, 2001). Using a micropipette to bend the primary cilium also resulted in a comparable decrease in $DiBAC_4(3)$ fluorescence (Fig. 1C), amounting to 7.27 ± 0.82% (n = 9).

Calibration of the E_M Change

$DiBAC_4(3)$ is one of the "slow" voltage-sensitive dyes that alter their distribution between cell membrane and cytoplasm as a function of E_M (Bräuner et al., 1984). Because the relative concentration of the dye in the cytoplasm is the measured variable, an absolute value of E_M cannot be determined from the fluorescence signal alone. In addition, dye redistribution or bleaching after the baseline fluorescence intensity, making it nearly impossible to use the dye to compare steady-state differences in E_M. However, transients in E_M can be faithfully detected and analyzed from the fluorescence, provided that the relationship between a change in fluorescence intensity and E_M has been determined experimentally. The fluorescence changes observed with $DiBAC_4(3)$ were calibrated by altering E_M by a predictable amount when the K^+ channels were assumed to be open, that is during the peak hyperpolarization of the E_M in response to flow. At this time point, the E_M measured previously with glass microelectrodes increased from the control value of −22.3 mV to −56.0 mV, approaching the

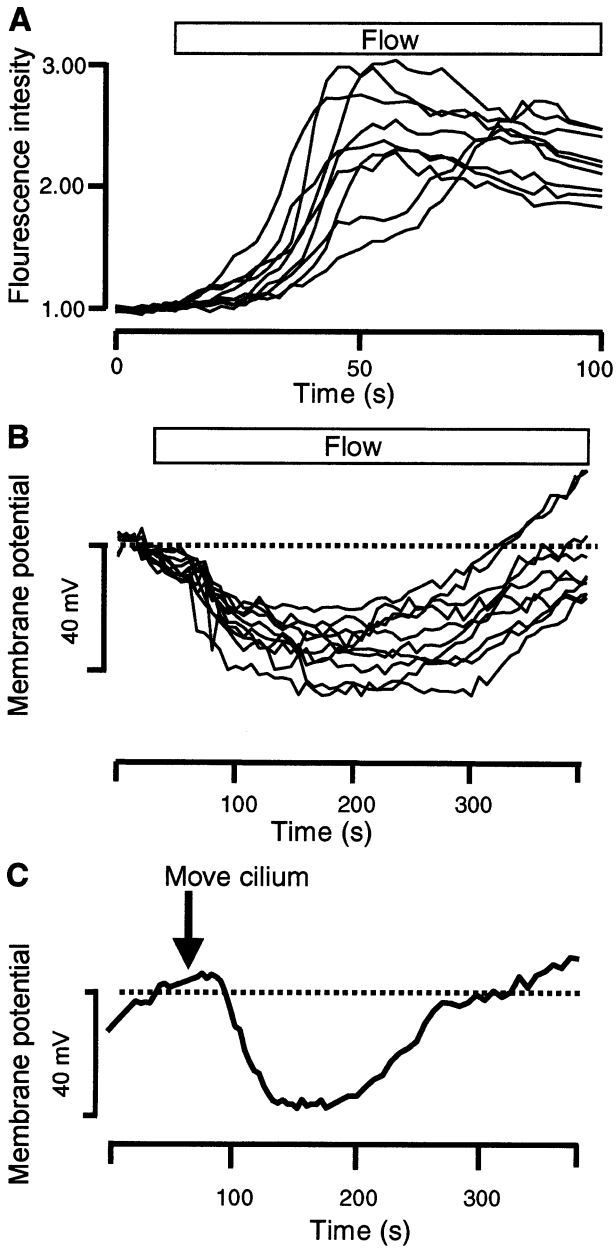

Fig. 1. (*A*) Changes in intracellular Ca^{2+} concentration as indicated by Fluo-4 fluorescence, when the perfusion rate is increased from 2 μl sec^{-1} to 8 μl sec^{-1}. (*B*) The effect of increasing the flow rate on the membrane potential (E_M) as indicated by $DiBAC_4(3)$ fluorescence. (*C*) The effect on the membrane potential of a single cell of bending its primary cilium with a micropipette.

calculated equilibrium potential for K^+, $E_K = -77$ mV (Praetorius & Spring, 2001).

A two-point calibration was performed with isosmotic extracellular K^+ concentrations either of 5.4 mM or 100 mM. Intracellular K^+ concentration was assumed to be 95 mM under steady-state conditions, based on the value of 94.8 mM reported for collecting duct (Sansom et al., 1989). When the equilibrium potential was calculated for the two concentrations according to the Nernst equation, a 1% ($\Delta F/F$) change of $DiBAC_4(3)$ fluorescence was equivalent to an E_M change of 3.83 mV. This correlation is comparable to that reported by Russ, Rauch & Quast (1999) for $DiBAC_4(3)$ fluorescence in renin-secreting cells from rat kidney glomeruli (1% $\Delta F/F = 4.0 \pm 0.3$ mV). Thus, the flow-induced hyperpolarization measured with the dye was 29 ± 1.2 mV, not significantly different from the value of 33.7 ± 3 mV obtained previously with glass microelectrodes (Praetorius & Spring, 2001). Assuming that the resting E_M is -22.3 mV, as measured previously, the flow-induced hyperpolarization measured with $DiBAC_4(3)$ would increase the E_M to -51.3 mV.

The good agreement between the voltage-sensitive dye and the microelectrode measurements of the transients in E_M gave us confidence in the dye. We proceeded to use the dye to test the relationship between the Ca^{2+} increase and the hyperpolarization.

Calcium Dependence of the Hyperpolarization

To test whether the flow-induced hyperpolarization was a consequence of the increase in intracellular Ca^{2+} concentration that follows bending the cilium, the flow experiments were repeated in the presence of 30 μM Gd^{3+}. We had previously shown that Gd^{3+} abolished the flow-induced increase in intracellular Ca^{2+} concentration in MDCK cells (Praetorius & Spring, 2001). As shown in Fig. 2A, Gd^{3+} completely eliminated the hyperpolarization in response to flow. The relative fluorescence of $DiBAC_4(3)$ measured 100 seconds after flow was increased from 2 to 8 μl sec^{-1} in the presence of Gd^{3+} was $-0.22 \pm 0.74\%$ ($n = 44$), equivalent to a voltage change of -2.3 ± 2.5 mV, not significantly different from zero.

Evidence for Potassium Channel Activation

Since the flow-induced hyperpolarization is Ca^{2+}-dependent and E_M moves toward E_K, we tested the effect of the K^+-channel blocker Ba^{2+}. Inclusion of 5 mM Ba^{2+} in the perfusate significantly reduced the magnitude of the flow-induced $DiBAC_4(3)$ fluorescence decrease to -3.78 ± 0.52 %, $n = 71$ (Fig. 2*B*). This corresponds to a 14.5 ± 2 mV hyperpolarization, bringing the estimated E_M to -37.8 mV. Because the E_M is the sum of all of the ion conductances and driving forces and Ba^{2+} reduced the hyperpolarization by only 50%, there must be another significant membrane conductance or electromotive force that tends to hold the E_M at -37.8 mV. Because we expected 5 mM Ba^{2+} to block all K^+ channels, we tested for the presence of a significant apical membrane conductance to Cl^-.

The E_M previously recorded with microelectrodes under control conditions was close to that calculated

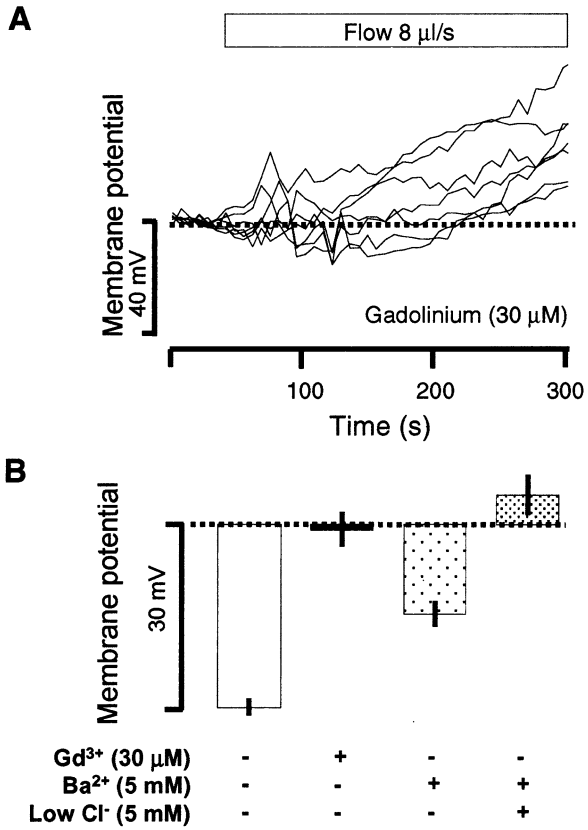

Fig. 2. (*A*) The inhibitory effect of Gd^{3+} (30 µM) on the flow-induced hyperpolarization. (*B*) The effects of Gd^{3+} (30 µM), Ba^{2+} (5 mM) and low external Cl^- (5 mM) on the maximal hyperpolarization induced by flow. Gd^{3+} (30 µM) or Ba^{2+} 5 mM) were added during the equilibration period with $DiBAC_4(3)$; the low-Cl^- solution (11.6 mM) was only applied when the flow on the apical side was increased. The bars (with SEM) represent the maximal hyperpolarization or, when no hyperpolarization was observed, the value at the time when the control flow-induced hyperpolarization was maximal. Total number of tested cells: control, $n = 150$; Gd^{3+}, $n = 44$; Ba^{2+}, $n = 71$; Ba^{2+} + low-Cl^-, $n = 45$.

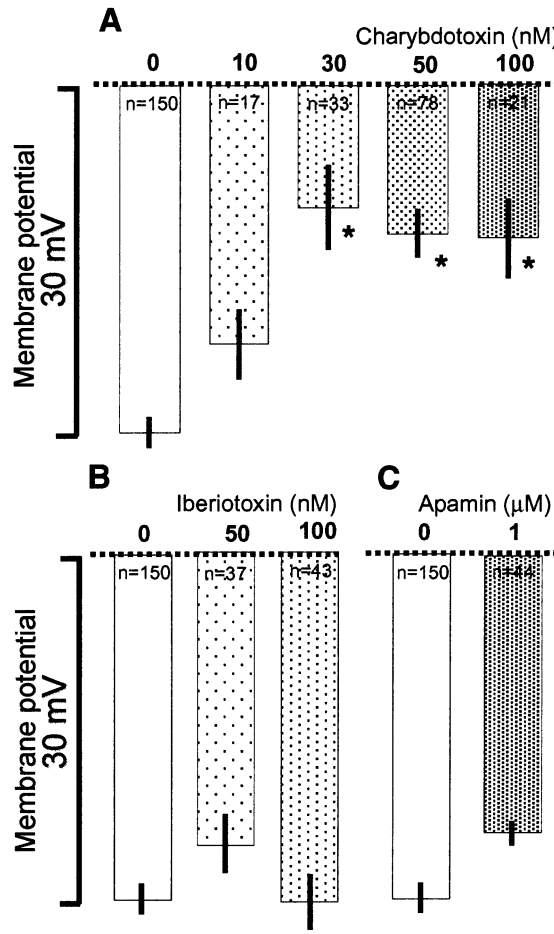

Fig. 3. The effect of inhibitors of Ca^{2+}-sensitive K^+ channels on the flow-induced hyperpolarization of MDCK cells measured 100 seconds after the initiation of flow. (*A*) Inhibition by charybdotoxin (0–100 nM), (*B*) Iberiotoxin (50 nM and 100 nM). (*C*) Aparnin (1 µM). Asterisks indicate values that differ significantly from control (concentration 0).

for the chloride equilibrium potential, $E_{Cl} = -20.4$ mV, using an intracellular Cl^- activity of 58 mM previously determined in MDCK cell cysts by ion-sensitive microelectrode, both in the presence and absence of HCO_3^- (Macias et al., 1992). To test for the presence of a Cl^- conductance when the cilia were bent by flow, the epithelium was perfused with a low Cl^- solution that also contained 5 mM Ba^{2+}. The cells were first incubated in a control solution containing 5 mM Ba^{2+}, then they were perfused with a solution in which the Cl^- concentration was reduced approximately tenfold to 11.6 mM. As shown in Fig. 2*B*, when the low-Cl^- solution reached the cell, $DiBAC_4(3)$ fluorescence increased relative to control, indicating that E_M moved in a depolarizing instead of a hyperpolarizing direction. The increase in $DiBAC_4(3)$ fluorescence amounted to 1.22 ± 0.84% ($n = 56$), equivalent to a depolarization of +4.2 ± 3.1 mV compared to the control E_M in the absence of Ba^{2+}. The estimated E_M became −18.1 mV as a consequence of the 19.7 mV depolarization, compared to the E_M in the presence of Ba^{2+} alone. Although this depolarization represents a change toward the calculated E_{Cl} of +42 mV, its magnitude is considerably less than the approximately 60 mV expected for a purely Cl^- elective membrane.

Because a K^+ current was the major component of the flow-induced hyperpolarization and the hyperpolarization was Ca^{2+}-dependent, we tested inhibitors of Ca^{2+}-sensitive K^+ channels (Fig. 3). Charybdotoxin, an inhibitor of maxi-K^+ channels as well as of intermediate-conductance Ca^{2+}-sensitive K^+ channels (Cai, Garneau & Sauve, 1999), clearly showed a dose-dependent inhibition of the flow-induced hyperpolarization (Fig. 3*A*). Maximal inhibition of 72.1% was seen with 30 nm charybdotoxin. This corresponds to a hyperpolarization of only 8 mV

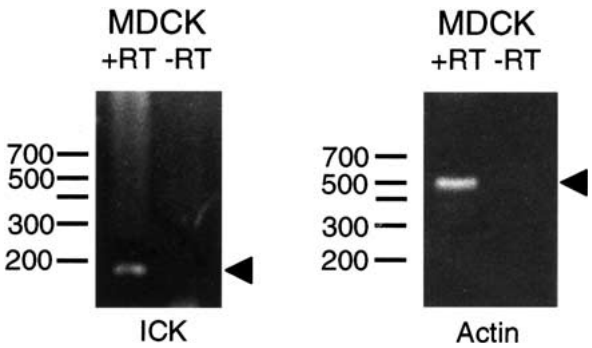

Fig. 4. Detection of mRNA encoding intermediate-conductance K^+ (ICK) channels in MDCK cells. Reverse transcription-polymerase chain reaction was performed on cDNA from wild-type MDCK Cells. The right panel shows that a PCR product of the expected molecular size (*arrowhead*) was obtained with primers specific for human and pig ICK channels only when reverse transcription was performed (+RT). Omission of reverse transcriptase (−RT) prevented this PCR product. Control PCR for actin revealed a PCR product of expected size (*arrow head*) only with reverse transcribed mRNA (+RT).

but constitutes a greater inhibition than was obtained with 5 mM Ba^{2+}. Iberiotoxin, an inhibitor of maxi-K^+ channels, but not of intermediate-conductance K^+ channels, failed to affect the hyperpolarization at concentrations as high as 100 μM. Apamin (1 μM), an inhibitor of small-conductance Ca^{2+}-activated K^+ channels, resulted in a small, but statistically significant ($p = 0.02$) decrease in the magnitude of the flow-induced $DiBAC_4(3)$ fluorescence change to 6.1 ± 0.3% ($n = 44$). This value was slightly less than the control hyperpolarization of 29 mV and was equivalent to a hyperpolarization of −23.4 ± 1.0 mV. All of the effects of the K^+ channel inhibitors were confirmed by transepithelial electrical resistance measurements of MDCK cell monolayers grown on permeable supports and studied in an Ussing chamber (*data not shown*). Thus, we conclude that an intermediate conductance K^+ channel opens in response to the flow-induced calcium increase and that most of the hyperpolarization can be eliminated by specific blockade of that channel.

RT-PCR and Sequencing

The presence of mRNA encoding intermediate-conductance K^+ channels in MDCK cells was investigated by RT-PCR. As shown in Fig. 4, a PCR product of expected ~165 bp was detected when cDNA from wild-type MDCK cells was used as template with primers specific for intermediate-conductance K^+ channels. Sequence analysis revealed that the 165 bp PCR product showed 100% nucleotide identity with the published sequence for intermediate-conductance K^+ channels from humans and pigs. Contamination by chromosomal DNA was absent, since the formation of the 165-bp band was dependent on reverse transcription. Furthermore, the intron-spanning actin primers produced bands corresponding to the transcribed sequence of the protein code, as shown Fig. 4. The sequence of the canine PCR product shared 94% nucleotide identity with the human intermediate-conductance Ca^{2+} activated K^+ channel (Genbank access # AF395661) and only one of 48 amino acids of the translated sequence differed from the corresponding human protein (Genbank access # AAK81862L).

Discussion

The main finding of this study is that Ca^{2+}-sensitive intermediate-conductance K^+ (IK^+) channels are activated by bending of the primary cilium either by micropipette or by flow in MDCK cells. The bending of the primary cilium results in an increase in the intracellular Ca^{2+} concentration that mediates the opening of the IK^+ channels, producing a substantial hyperpolarization of the membrane. In a recent study, we showed that the presence of primary cilia is essential for sensing changes in apical flow rates (Praetorius & Spring, 2003). The primary cilium, therefore, constitutes the critical link between flow and electrical events. The voltage-sensitive fluorescent dye $DiBAC_4(3)$ appeared to faithfully reproduce both the time course and the magnitude of the changes in E_M previously obtained with microelectrodes, while eliminating most of the technical difficulties associated with their use.

Comparison of the time courses of the Fluo-4 and $DiBac_4(3)$ traces shows that the peak hyperpolarization lags about 36 seconds behind the maximal increase in Ca^{2+}. This temporal correspondence, coupled with the complete inhibition of the hyperpolarization by Gd^{3+}, an inhibitor that is known to prevent the Ca^{2+} response to bending the cilium, supports the conclusion that the change in E_M is a direct consequence of the large increase in intracellular Ca^{2+}. The time interval between the maximal increase in intracellular Ca^{2+} and the peak hyperpolarization is remarkably long. The lag time cannot be accounted for by the slowness of the potential-sensitive probe, since a similar lag period was observed when the membrane potential was measured with microelectrodes (Praetorius & Spring, 2001). Although we did not address this issue, we speculate that K^+ channel activation may require a sequence of events involving the cytoskeleton, as was recently reported for the rat cortical collecting duct (Wei and Wang, 2002).

Like the native collecting duct from which it was derived, wild-type MDCK cell cultures consist of both primary cilia expressing principal cells and non-ciliated intercalated cells (Gekle et al., 1994). It is the

MDCK principal cells that sense alteration in apical flow by the use of their primary cilium (Praetorius & Spring, 2001, 2002) and that respond with increases in the intracellular Ca^{2+} concentration over an extended period of time and with a concomitant hyperpolarization of the apical E_M. Increasing intracellular Ca^{2+} concentration by means of a Ca^{2+} ionophore has also been shown to produce a transient hyperpolarization of a few minutes duration in MDCK cells (Breuer et al., 1988), although the cell type was not determined. In the present investigation, we confined our measurements to ciliated principal cells.

Membrane Potential in MDCK Cells

We previously reported that the E_M of control MDCK cells is -22.3 ± 2.7 mV, in good agreement with the $E_M = -29 \pm 4$ mV measured by Macias et al. (1992) in MDCK cell cysts in the absence of HCO_3^-. Breuer et al. (1988) reported an E_M of -18 to -25 mV for dissociated MDCK ceils in HEPES-buffered solution, using a voltage-sensitive oxonol dye. Macias et al. (1992) showed that E_M in the presence of HCO_3^- was -50 ± 2 mV, considerably more negative than it its absence. The control value in the absence of HCO_3^- in their study and that in our previous investigation (Praetorius & Spring, 2001) is very close to the predicted E_{Cl} of -20.4 mV, consistent with the conclusion that there is little or no resting K^+ conductance of the apical membrane in HEPES solutions. This agrees with a previous report that MDCK cell E_M was unaffected by an increased K^+ concentration in the apical perfusate (Husted, Welsh & Stokes, 1986).

Although our present experiments are consistent with the presence of a significant Cl^- conductance in the apical membrane of MDCK cells, both under control and flow-stimulated conditions, it is uncertain whether there is activation of additional Cl^- conductance by flow. The E_M under control conditions is very close to E_{Cl}. After flow stimulation, the E_M moves toward E_K and blockade of the K^+ conductance by charybdotoxin results in an E_M that is again near E_{Cl}. Thus, even if there were activation of a Cl^- conductance by the increased Ca^{2+}, it still would not result in the observed hyperpolarization. The experiments with a low-Cl^- perfusate showed that E_M remained far from E_{Cl} and that there must be additional conductance or electromotive forces contributing to E_M. It has previously been shown that flow activates the Na^+/K^+ ATPase in collecting duct (Giebisch, 1998); such activation of the pump would result in a hyperpolarization of the membrane. Because our attention was directed toward determining the nature of the K^+ conductance induced by the flow-induced Ca^{2+} increase, we did not investigate this point further.

Flow and K^+ Secretion

The collecting duct is known to increase its rate of K^+ secretion as lumenal flow rate rises (Giebisch, 1998). The principal cells of the epithelium are responsible for this secretion through K^+ channels in the apical membrane (Stanton et al., 1981, Koeppen, Biagi & Giebisch, 1983; Sansom & O'Neil, 1985). The amount of K^+ secretion is determined by the electrochemical gradient for K^+ across the apical membrane and by the open-state probability of the K^+ channels, which is subject to modulation by the intracellular Ca^{2+} concentration (Giebisch, 1998). Collecting ducts were previously reported to express small-conductance K^+ channels in the apical membrane (Frindt & Palmer, 1989). On the basis of their sensitivity to charybdotoxin and insensitivity to apamin, it was recently concluded that maxi-K^+ channels are the primary mediators of flow-dependent potassium secretion in the collecting duct (Tanigushi & Imai, 1998; Woda et al., 2001). However, discrimination between the maxi-K^+ and intermediate-conductance K^+ channels cannot be made solely on the basis of inhibition by charybdotoxin, but requires the use of another selective agent such as iberiotoxin (Cai, Garneau & Sauve, 1998).

MDCK cells have been reported to express two types of Ca^{2+}-sensitive K^+-channels: a non-rectifying maxi K^+ channel (Bolivar & Cererijido, 1987), and an intermediate-conductance K^+ channel (IK^+) (Friedrich et al., 1988, Schwab et al., 1993). Testing various blockers of Ca^{2+}-activated K^+-channels revealed that the flow-induced hyperpolarization was inhibited only by charybdotoxin. On the other hand, the Ca^{2+}-activated K^+ channel blockers iberiotoxin and apamin had no effect on the flow-induced hyperpolarization. Charybdotoxin is known to inhibit both maxi K^+ channels and IK^+ channels (Cai et al., 1998), but recently, the combination of charybdotoxin and iberiotoxin has been used to pharmacologically characterize IK^+ channels from migrating transfected MDCK cells (Wulf & Schwab, 2002). Here, we show that the wild-type MDCK cells, like the migration-transformed MDCK cell (Wulf & Schwab, 2002), express mRNA encoding intermediate-conductance K^+ channels. The present observations suggest a function for the IK^+ channels not only in spreading, migrating cells, but also in the mature, confluent MDCK cells.

It is of interest that charybdotoxin was considerably more effective than Ba^{2+} blocking the hyperpolarization. The diminished effectiveness of Ba^{2+} may be related to the low E_M, as Ba^{2+} is known to be a voltage-dependent inhibitor of K^+ channels in cortical collecting duct cells (O'Niel, 1983). However, since charybdotoxin did not completely eliminate the hyperpolarization, while Gd^{3+} did, there must be another Ca^{2+}-sensitive electromotive force that ac-

counts for the residual 8 mV hyperpolarization in the presence of charybdotoxin.

The Ca^{2+}-induced increase in K^+ channel activity in MDCK cells does not provide a full explanation for flow-induced K^+ secretion in renal tubules. As the flow-induced Ca^{2+} response both in MDCK cells (Praetorius & Spring, 2001) and in rabbit collecting ducts (Woda et al., 2002) is known to be transient, another mechanism must be activated to sustain the flow-induced K^+ secretion observed in collecting duct. The channel type responsible for the K^+ secretion in the collecting duct is likely to be the Maxi K^+ channel (Woda et al., 2001) rather than the IK^+ channels seen in MDCK cells. We speculate that an additional second messenger, which doesn't affect the IK^+ channels of the MDCK cells, might maintain the Maxi K^+-channel activity in the collecting duct.

In summary, we conclude that fluid perfusion of the apical surface of MDCK cells elevates intracellular Ca^{2+} concentration because the flowing solution bends the primary cilium and activates Ca^{2+} entry through mechanosensitive channels in the cilium. The increase in intracellular Ca^{2+} concentration that follows activates IK^+ channels in the apical membrane, leading to a substantial hyperpolarization of the membrane. This sequence of events provides a foundation for understanding the flow dependence of K^+ secretion and transepithelial potential difference in the collecting duct.

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