

# TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity

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**A**utoregulation of the ciliary beat frequency (CBF) has been proposed as the mechanism used by epithelial ciliated cells to maintain the CBF and prevent the collapse of mucociliary transport under conditions of varying mucus viscosity. Despite the relevance of this regulatory response to the pathophysiology of airways and reproductive tract, the underlying cellular and molecular aspects remain unknown. Hamster oviductal ciliated cells express the transient receptor potential vanilloid 4 (TRPV4) channel, which is activated by increased viscous load involving a phospholipase A<sub>2</sub>-dependent pathway.

TRPV4-transfected HeLa cells also increased their cationic currents in response to high viscous load. This mechanical activation is prevented in native ciliated cells loaded with a TRPV4 antibody. Application of the TRPV4 synthetic ligand 4 $\alpha$ -phorbol 12,13-didecanoate increased cationic currents, intracellular Ca<sup>2+</sup>, and the CBF in the absence of a viscous load. Therefore, TRPV4 emerges as a candidate to participate in the coupling of fluid viscosity changes to the generation of the Ca<sup>2+</sup> signal required for the autoregulation of CBF.

## Introduction

Epithelial ciliated cells are responsible for the mechanical clearance of mucus and trapped substances from the airways and the transport of gametes and embryos through the oviduct (Halbert et al., 1976; Afzelius, 1995; Knowles and Boucher, 2002). A primary determinant of mucociliary transport is the ciliary beat frequency (CBF), which is regulated by a variety of chemical and mechanical stimuli (Satir and Sleight, 1990). Ciliated epithelia are exposed to physiological changes in mucus viscosity (Rutllant et al., 2002). Despite these variations in fluid viscosity, mucociliary transport efficiency is preserved. Johnson et al. (1991) have shown that ciliated cells are able to maintain relatively constant their CBF over a range of viscosities and proposed that this autoregulatory response of the CBF aimed to prevent the collapse of mucus transport under high viscous loads.

Several intracellular signals have been proposed to mediate the changes of CBF in response to different stimuli: cAMP, cGMP, nitric oxide, and Ca<sup>2+</sup> (Jain et al., 1993; Geary et al., 1995; Wyatt et al., 1998; Evans and Sanderson, 1999). Among

them, the role of Ca<sup>2+</sup> in the control of CBF is particularly interesting as it has been associated with the ciliary response to mechanical stimuli. Mechanically stimulated ciliated cells increase intracellular Ca<sup>2+</sup> and CBF (Lansley and Sanderson, 1999), a response that is lost in the absence of extracellular Ca<sup>2+</sup> (Sanderson and Dirksen, 1986). The hypothesis that mechanical stimulation might be physiologically initiated by changes in mucus viscosity has been present for quite a while (Spungin and Silberberg, 1984), but the cellular mechanism linking the viscous load exerted by the presence of mucus to the control of CBF awaits to be resolved. In the present work, we aimed to elucidate the mechanism that couples mechanical stimulation (viscous load) to ciliary activity, a process that has been suggested to involve Ca<sup>2+</sup> entry and subsequent activation of cilia (Spungin and Silberberg, 1984).

Over the past few years great advances have been made on the molecular characterization of the Ca<sup>2+</sup> entry pathways activated in response to different stimuli, and a new class of calcium-permeable cationic channels, the transient receptor potential (TRP) superfamily, has emerged (Clapham, 2003). The vertebrate TRPV4 channel has been proposed as an osmo- and mechanosensitive channel (Liedtke et al., 2000, 2003; Strotmann et al., 2000; Wissenbach et al., 2000; Nilius et al., 2001; Arniges et al., 2004). Here, we report the role of TRPV4 and phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in the generation of the Ca<sup>2+</sup>

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Abbreviations used in this paper: 4 $\alpha$ PDD, 4 $\alpha$ -phorbol 12,13-didecanoate; AACOCF<sub>3</sub>, arachidonyl trifluoromethyl ketone; CBF, ciliary beat frequency; NMDG, N-methyl-D-glucamine; pBPB, 4-bromophenacyl bromide; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; TRPV4, transient receptor potential vanilloid 4.

signal required to maintain CBF in hamster oviductal ciliated cells under conditions of mechanical stress induced by high viscous load, thereby preventing the collapse of the mucus transport.

## Results and discussion

### High viscosity-induced $\text{Ca}^{2+}$ -dependent autoregulation of the CBF in oviductal ciliated cells

Exposure of primary cultures of hamster oviductal ciliated cells to increased viscous loading reduced the CBF, reaching a new stable value within the first 10 min (Fig. 1 a). The CBF dropped  $\sim 35\%$  within the range of 2–37 cP (2–15% dextran solutions), but no further decrease was observed at higher viscosities in the range of 37–200 cP (15–30% dextran solutions; Fig. 1 b). These results indicate that mucus transporting ciliated cells are capable of maintaining their CBF in high viscosity conditions and suggest the presence of an autoregulatory mechanism that allows ciliated epithelia to adjust their CBF to varying viscous loads without collapsing mucus transport. The signal coupling changes in mechanical load (fluid viscosity) to the autoregulation of CBF is still unknown, although early works pointed to the influx of  $\text{Ca}^{2+}$  into the cells as a probable candidate (Johnson et al., 1991). We tested the  $\text{Ca}^{2+}$  hypothesis by measuring CBF in ciliated cells exposed to either 5% (4.8 cP) or 20% dextran solutions (73 cP, viscosity value within the range where autoregulation of the CBF occurred) in the absence of extracellular  $\text{Ca}^{2+}$  or in the presence of 100  $\mu\text{M}$   $\text{Gd}^{3+}$ , a blocker of mechanosensitive cation channels (Yang and Sachs, 1989). Fig. 1 c shows that neither the absence of extracellular  $\text{Ca}^{2+}$  or the presence of  $\text{Gd}^{3+}$  modified the CBF at low viscosity conditions but determined a marked reduction of the CBF at high viscous loads (73 cP; Fig. 1 d). These results suggested that the autoregulation of CBF at high viscous loads required the entry of  $\text{Ca}^{2+}$  into the ciliated cell, a process that apparently does not play a crucial role in the establishment of the steady-state CBF at lower viscosities.

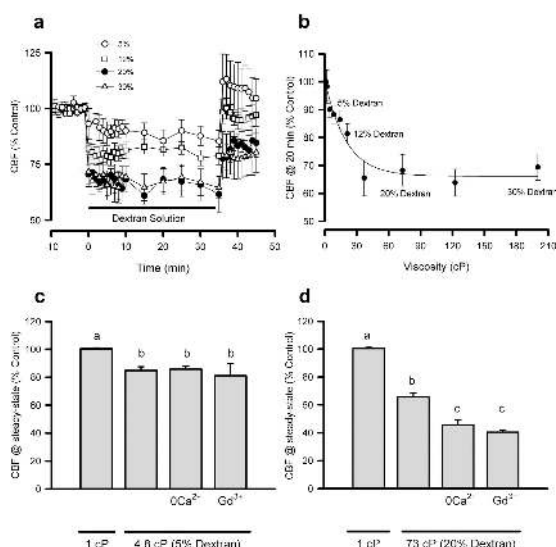
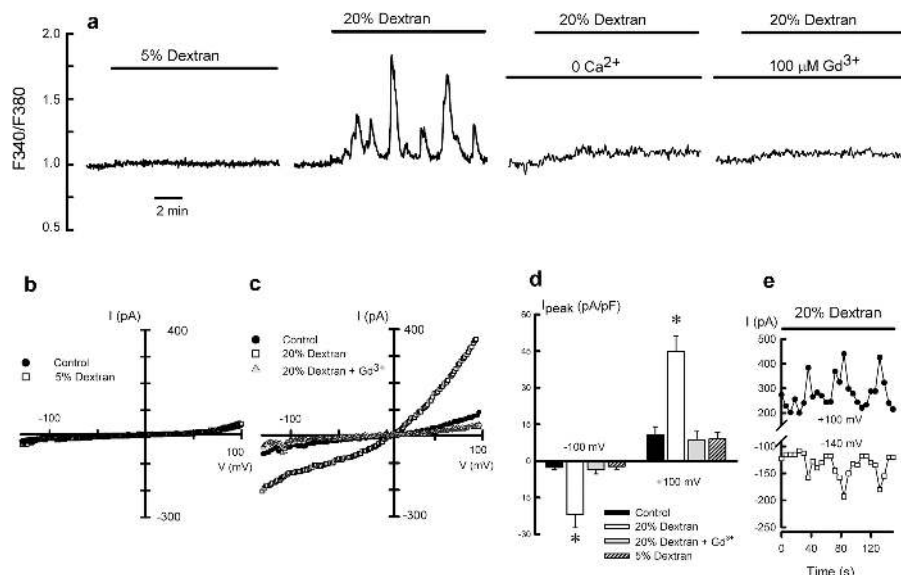


Figure 1. **Effect of viscous loading on CBF.** (a) Time course of CBF changes in hamster oviduct ciliated cells exposed to 5% (4.8 cP), 12% (21.5 cP), 20% (73 cP), and 30% (200 cP) dextran solutions. (b) Effect of viscous load on the CBF recorded after 20-min exposure to increased viscosity. CBF recorded at steady-state conditions (15–25 min) after exposure to 4.8 (c) or 73 cP (d) in the absence of extracellular  $\text{Ca}^{2+}$  or in the presence of 100  $\mu\text{M}$   $\text{Gd}^{3+}$ . Results are the mean  $\pm$  SEM of 5–10 separate cultures. Significant differences ( $P < 0.05$ ) between groups are marked with different letters.

To further investigate the relationship between mechanical stimulation and intracellular  $\text{Ca}^{2+}$ , we monitored changes in cytosolic  $\text{Ca}^{2+}$  in response to low (5% dextran) and high (20% dextran) viscous loads, the latter triggering the CBF autoregulatory response. Fig. 2 a shows the ratiometric fura-2 fluorescence obtained in primary cultures of single oviductal ciliated cells. Exposure to 5% dextran solution did not modify the intracellular  $\text{Ca}^{2+}$  levels (Fig. 2 a), whereas 20% dextran solution (73 cP) triggered an oscillatory  $\text{Ca}^{2+}$  response in ciliated cells, which was prevented in the absence of extracellular  $\text{Ca}^{2+}$  or in the presence of 100  $\mu\text{M}$   $\text{Gd}^{3+}$ . Interestingly, the  $\text{Ca}^{2+}$  oscillations

### Figure 2. Dextran-activated calcium entry pathway.

(a) Cytosolic  $\text{Ca}^{2+}$  signal obtained in hamster oviductal ciliated cells exposed to 5 or 20% dextran and the effect of extracellular  $\text{Ca}^{2+}$  removal and 100  $\mu\text{M}$   $\text{Gd}^{3+}$  on the 20% dextran-induced  $\text{Ca}^{2+}$  signal. Traces are representative of five to six experiments under each condition. (b and c) Whole-cell cationic currents recorded in oviductal ciliated cells dialyzed with CsCl-containing pipette solution under control (1 cP) and 5% dextran (4.8 cP) (b) and 20% dextran solution alone (73 cP) or containing 100  $\mu\text{M}$   $\text{Gd}^{3+}$  (c). (d) Average current density measured at  $-100$  mV and  $+100$  mV under the following conditions: control ( $n = 27$ ), 20% dextran ( $n = 15$ ); 20% dextran +  $\text{Gd}^{3+}$  ( $n = 10$ ); and 5% dextran ( $n = 11$ ). \*,  $P < 0.05$ , compared with control. (e) Oscillatory pattern of the cationic current obtained in a single cell exposed to 20% dextran.



tory pattern emerged at the viscosity values that trigger the autoregulatory process ( $>30$  cP; unpublished data).

### Activation of a cationic current by high viscous load

The TRPV4 channel has been implicated in the cellular response to different mechanical stimuli (Liedtke et al., 2003; Suzuki et al., 2003); therefore, constituting a possible candidate to mediate  $\text{Ca}^{2+}$  entry in oviductal ciliated cells subjected to a high viscous load. Characterization of the putative TRPV4 cationic currents in response to increases in viscous load was evaluated in freshly dissociated single ciliated cells using the whole-cell patch-clamp technique. Exposure of a ciliated cell to a 5% dextran solution (4.8 cP) failed to activate a cationic current (Fig. 2 b), whereas exposure to a 20% dextran solution (73 cP) activated a large, outwardly rectifying current (Fig. 2 c), which was blocked by  $\text{Gd}^{3+}$  (100  $\mu\text{M}$ ). Mean normalized peak current responses to 5 and 20% dextran solutions are shown in Fig. 2 d. The TRPV4-like current generated in response to 20% dextran often presented an oscillatory pattern (Fig. 2 e) resembling the  $[\text{Ca}^{2+}]_i$  oscillations shown in Fig. 2 a.

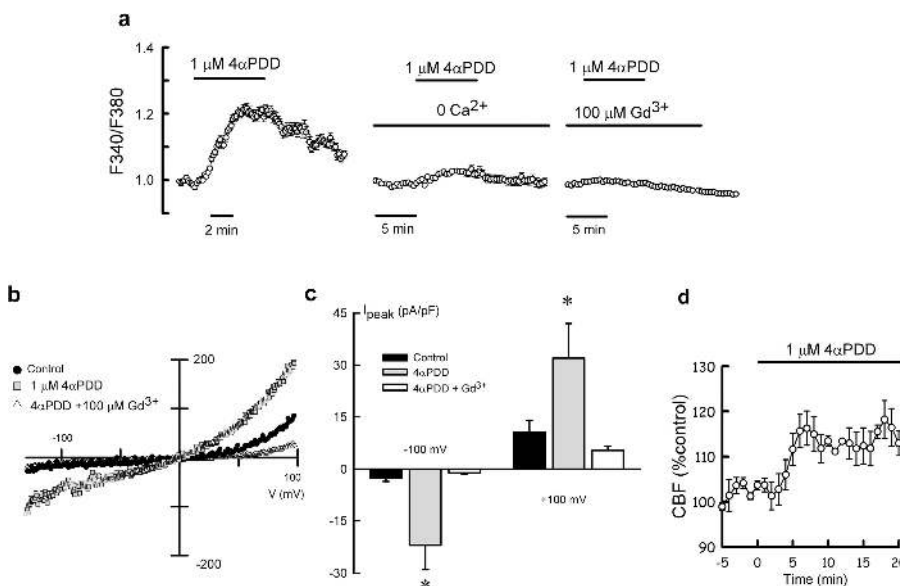
Functional TRPV4 channels in oviductal ciliated cells were also evaluated using a synthetic activator of TRPV4, 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ PDD; Watanabe et al., 2002), in the absence of viscous load (1 cP). Intracellular  $\text{Ca}^{2+}$  in ciliated cells increased in response to 4 $\alpha$ PDD (Fig. 3 a), and, occasionally, showed an oscillatory pattern (not depicted). This response was prevented in the absence of extracellular  $\text{Ca}^{2+}$  (Fig. 3 a, middle) or in the presence of  $\text{Gd}^{3+}$  (Fig. 3 a, right), a blocker of nonselective cation channels. A whole-cell current with characteristics similar to that obtained with 20% dextran solutions was recorded when ciliated cells were exposed to 1  $\mu\text{M}$  4 $\alpha$ PDD (Fig. 3, b and c). Analysis of the reversal potential of the currents activated by 20% dextran solutions ( $-4.7 \pm 2.4$  mV;  $n = 15$ ) and 4 $\alpha$ PDD ( $-5.5 \pm 2.4$  mV;  $n = 6$ ) are not statistically different but showed a right shift compared with con-

trol currents ( $-12 \pm 1.3$  mV;  $n = 21$ ) as previously described (Watanabe et al., 2002). Addition of 4 $\alpha$ PDD also resulted in the increase of the CBF (Fig. 3 d) in the absence of mechanical stimuli (1 cP), thus adding support to the hypothesis linking the activity of TRPV4 channels to the control of CBF at high viscous loads. Changes in flow superfusion (1–15 ml/min) in the absence of dextran did not activate cationic currents, whereas exposure to 20% dextran solutions activated them either in the absence of fluid flow or under continuous perfusion at 1–3 ml/min. Therefore, it appears that high viscous load by itself is the main trigger of the response, although we cannot completely discard a shear stress component in the response elicited by high dextran solutions.

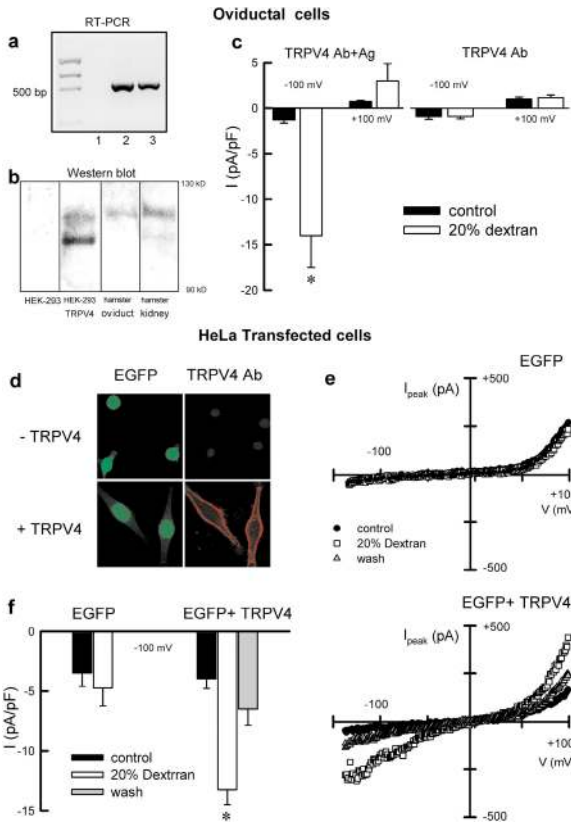
### Molecular identification of the dextran-induced cationic currents

All the functional data shown point to a TRPV4-like channel as the mediator in the increased  $[\text{Ca}^{2+}]_i$  required for the CBF autoregulatory response. Molecular identification of TRPV4 in hamster oviductal ciliated cells was investigated by single-cell RT-PCR and Western blot. Hamster TRPV4 has not yet been cloned, so we used primers directed against a region of the TRPV4 sequence highly conserved across species. Fig. 4 a shows single amplicons of  $\sim 500$  bp obtained from two hamster oviductal ciliated cells. Subsequent sequencing of the bands confirmed the expression of TRPV4 in the ciliated cells. Fig. 4 b shows a Western blot obtained with an antibody generated against a COOH-terminal epitope of the human TRPV4 protein. The antibody identified a double band of the expected size in human TRPV4-transfected HEK cells and a single band in hamster oviductal cells.

Next, we functionally tested the blocking capability of the TRPV4 antibody on oviductal cells to confirm that a TRPV4-like channel is at the core of the ciliated cells' response to high viscous loads. Intracellular dialysis of hamster oviductal ciliated cells with the TRPV4 antibody prevented the activation of cationic currents under high viscous



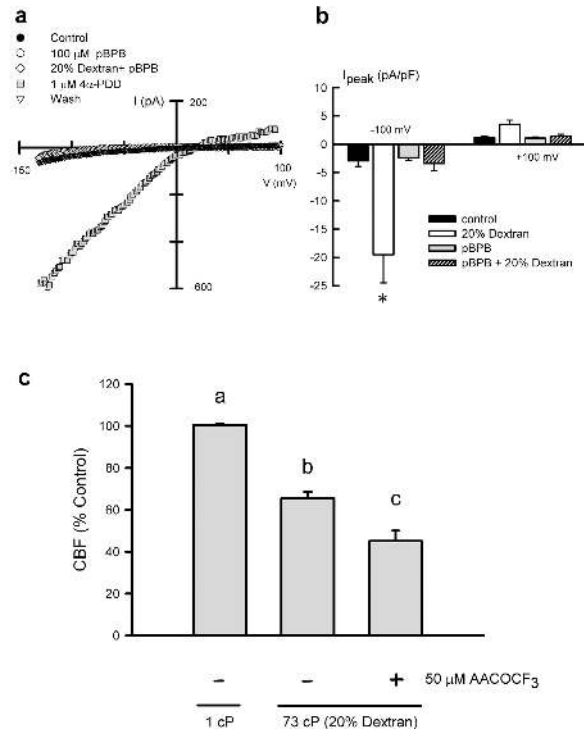
**Figure 3. Effect of the TRPV4 activator 4 $\alpha$ PDD on cytosolic calcium, cationic currents, and CBF.** (a, left) Cytosolic  $\text{Ca}^{2+}$  response of ciliated oviductal cells to 4 $\alpha$ PDD (1  $\mu\text{M}$ ). Effect of removal of extracellular  $\text{Ca}^{2+}$  (middle) and 100  $\mu\text{M}$   $\text{Gd}^{3+}$  (right) on the 4 $\alpha$ PDD response. Traces are means  $\pm$  SEM obtained from 6–16 cells per culture (repeated on at least three cultures). (b) Whole-cell cationic currents recorded in an oviductal ciliated cell dialyzed with CsCl-containing pipette solution under control conditions, after application of 1  $\mu\text{M}$  4 $\alpha$ PDD, and after application of 1  $\mu\text{M}$  4 $\alpha$ PDD + 100  $\mu\text{M}$   $\text{Gd}^{3+}$ . (c) Average current density measured at  $-100$  mV and  $+100$  mV under the following conditions: control ( $n = 15$ ), 1  $\mu\text{M}$  4 $\alpha$ PDD ( $n = 6$ ), and 4 $\alpha$ PDD + 100  $\mu\text{M}$   $\text{Gd}^{3+}$  ( $n = 3$ ). \*,  $P < 0.05$ , compared with control. (d) Time course of CBF response to 1  $\mu\text{M}$  4 $\alpha$ PDD ( $n = 6$ ).



**Figure 4. Molecular identification of dextran-activated cationic currents.** (a) Detection of TRPV4 in two oviductal ciliated cells (lanes 2 and 3) by RT-PCR. (lane 1) Negative control where the cDNA was omitted. (b) Western blot showing bands at the predicted molecular mass for TRPV4 (~100 kD) in hamster oviduct and kidney. Untreated HEK-293 and HEK-293 cells transfected with the human TRPV4 (isoform a) were used as negative and positive controls, respectively. (c) Mean current density recorded in oviduct ciliated cells dialyzed with NMDG-Cl solutions containing either 3.2  $\mu\text{g/ml}$  (dilution 1:500) of TRPV4 antibody preabsorbed with a 10-fold excess of antigen (left;  $n = 5$ ) or TRPV4 antibody alone (right;  $n = 6$ ). Cells were superfused with 20% dextran solutions 10 min after the establishment of the whole-cell configuration. (d) Confocal images of HeLa cells transfected with EGFP (top) or EGFP+TRPV4 (bottom). EGFP fluorescence signal (green) and TRPV4 signal (red) are shown. (e) Whole-cell cationic currents recorded in HeLa cells with CsCl-containing pipette solution under control (1 cP), 20% dextran solution (73 cP), and washout. (f) Average current density measured at  $-100$  mV in HeLa cells transfected with EGFP ( $n = 9$ ) or EGFP+TRPV4 ( $n = 11$ ). \*,  $P < 0.05$ , compared with control.

load conditions (Fig. 4 c, right). The specificity of the inhibitory effect of the TRPV4 antibody was demonstrated by repeating the experiments with antigen-preabsorbed TRPV4 antibody. Under these conditions, normal activation of TRPV4-like currents were observed in response to 20% dextran solutions (Fig. 4 c, left).

The activation of TRPV4 by dextran was also tested in a heterologous expression system. Immunolocalization of TRPV4 to the plasma membrane was confirmed in transiently cotransfected (EGFP+hTRPV4; Fig. 4 d, bottom) but not in EGFP-transfected HeLa cells (Fig. 4 d, top). Exposure to 20% dextran solutions activated cationic currents only in EGFP+TRPV4-transfected cells (Fig. 4 e). Mean normalized currents obtained from EGFP- and EGFP+TRPV4-transfected HeLa cells are shown in Fig. 4 f.



**Figure 5. PLA<sub>2</sub>-dependent activation of TRPV4 under high viscous conditions.** (a) Whole-cell currents recorded in a cell dialyzed with NMDG-Cl solutions and bathed consecutively in control solutions, 100  $\mu\text{M}$  pBPB, 20% dextran + pBPB, and 1  $\mu\text{M}$  4 $\alpha$ PDD. Basal current levels recovered after washout. (b) Average current density measured at  $-100$  mV and  $+100$  mV with NMDG-Cl-containing pipette solutions and  $\text{Ca}^{2+}$ -free bathing solution under the following conditions: control ( $n = 16$ ), 20% dextran ( $n = 16$ ), pBPB ( $n = 12$ ), and pBPB + 20% dextran ( $n = 12$ ). \*,  $P < 0.05$ , compared with control. (c) CBF recorded at steady-state conditions (15–25 min) under control (1 cP) and 20% dextran solutions (73 cP) in the absence or presence of 50  $\mu\text{M}$  AACOCF<sub>3</sub>. Results are the mean  $\pm$  SEM of 5–10 separate cultures. Significant differences ( $P < 0.05$ ) between groups are marked with different letters.

### Involvement of PLA<sub>2</sub> in the autoregulation of the CBF

Cell swelling-dependent activation of TRPV4 requires the PLA<sub>2</sub>-mediated release of arachidonic acid (Vriens et al., 2004), suggesting that the TRPV4 channel is not an osmosensor per se, but a key element in the transduction of osmotic changes into  $\text{Ca}^{2+}$  signals. Both cell swelling and mechanical stress activate PLA<sub>2</sub> and the formation of arachidonic acid (Lehtonen and Kinnunen, 1995; Pedersen et al., 2000). Therefore, we evaluated whether or not activation of the TRPV4-like current by 20% dextran solutions might also depend on the activity of PLA<sub>2</sub> (Fig. 5). Currents were recorded with *N*-methyl-D-glucamine (NMDG) intracellular solutions, favoring the presence of inward currents. Incubation of single ciliated cells with the PLA<sub>2</sub> inhibitor 4-bromophenacyl bromide (pBPB; 100  $\mu\text{M}$ ) prevented the activation of TRPV4-like currents under high viscosity conditions (Fig. 5, a and b) but did not affect current activation by 4 $\alpha$ PDD (Fig. 5 a). These results are in accordance with previous observations reporting that swelling- and 4 $\alpha$ PDD-induced activation of TRPV4 use distinct pathways (Arniges et al., 2004; Vriens et al., 2004). Moreover, inhibition of PLA<sub>2</sub> activity with arachidonyl trifluoromethyl ketone

(AACOCF<sub>3</sub>; 50 μM) also prevented the cilia autoregulatory response. In the presence of AACOCF<sub>3</sub>, the CBF in response to 20% dextran solutions (73 cP) dropped to 44% of the basal value (Fig. 5 c), similar to the CBF reduction observed in the absence of Ca<sup>2+</sup> or in the presence of Gd<sup>3+</sup> (Fig. 1 d).

In conclusion, our findings offer a first insight into the molecular basis of the mechanotransduction process required for the maintenance of CBF in high viscosity conditions. Mechanical stimulation of ciliated cells, physiologically achieved by changes in mucus viscosity, activates a TRPV4-like channel that elevates intracellular Ca<sup>2+</sup>. Channel opening requires the activity of PLA<sub>2</sub> and occurs at high viscous loads; therefore, adapting cilia activity to a wide range of viscosities. Our results propose TRPV4 as a new target to consider in order to develop treatments for pathologies with altered mucociliary transport, including infertility and chronic respiratory diseases (Clarke, 1989; Afzelius, 1995).

## Materials and methods

### Chemicals and solutions

All chemicals were purchased from Sigma-Aldrich except dextran T-500 (500,000 D; Amersham Biosciences), fura-2AM (Molecular Probes), and AACOCF<sub>3</sub> (Calbiochem). Bathing solutions used for CBF and intracellular Ca<sup>2+</sup> measurements contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, 10 mM Hepes, pH 7.4, and 300 mosmol/Kg. Ca<sup>2+</sup>-free solutions were obtained replacing Ca<sup>2+</sup> by Mg<sup>2+</sup> and adding 5 mM EGTA. The viscosity of the bathing solution, a measure of the internal friction of a fluid (defined as the ratio = shear stress/shear rate), was increased by adding dextran without altering the solution's osmolality. 30% dextran solutions required a reduction of NaCl concentration (which did not affect CBF; Johnson et al., 1991) to maintain osmolality constant at 300 mosmol/Kg. Viscosity was measured using a cone-and-plate viscometer, and all the solutions used in this study behaved as Newtonian fluids (Johnson et al., 1991). After the exchange of the bathing solution by dextran solutions, the perfusion was stopped and the recordings were initiated. Alternatively, dextran solutions were superfused at a continuous flow rate of 1–3 ml/min.

### Primary cultures of Golden Hamster oviductal ciliated cells

Primary cultures were obtained and maintained as described previously (Hermoso et al., 2001) and single cells were isolated by enzymatic treatment of oviductal samples using a method previously described (Lock and Valverde, 2000). Primary cultures were used when a monolayer of ciliated cells showed spontaneous ciliary activity (5–7 d) and single cell preparations were used within 48 h. Animals were maintained and experiments performed according to the guidelines issued by the Institutional Ethics Committees of the Institut Municipal d'Investigació Mèdica (Universitat Pompeu Fabra) and the Pontificia Universidad Católica de Chile.

### Measurement of CBF and intracellular Ca<sup>2+</sup>

CBF was recorded using a microphotodensitometric technique (Hermoso et al., 2001). Hamster oviductal ciliated cells in culture beat spontaneously at 12.4 ± 0.9 Hz (mean ± SEM; n = 24). CBF data are expressed as a percentage of basal CBF at 1 cP (mean ± SEM). Cytosolic Ca<sup>2+</sup> was determined at 30–37°C in cells loaded with 5 μM fura-2AM using a spectrofluorometric (Hermoso et al., 2001) or ratio-imaging technique (Fernández-Fernández et al., 2002). Cytosolic Ca<sup>2+</sup> increases are presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm relative to baseline.

### Electrophysiology

Ionic currents were recorded in the whole-cell patch-clamp mode (Fernández-Fernández et al., 2002). Patch pipettes were filled with a solution containing 140 mM NMDG-Cl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM Hepes, 4 mM ATP, and 0.1 mM GTP, or 20 mM CsCl, 100 mM CsAcetate, 1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 10 mM Hepes, 4 mM ATP, and 0.1 mM GTP (300 mosmol/l, pH 7.3). Cells were held at 0 mV and ramps from –140 mV to +100 mV (400 ms) were applied at a frequency of 0.2 Hz. Ramp

data were acquired at 2 kHz and low-pass filtered at 1 kHz. Experiments were performed at RT (22–26°C).

### Single-cell RT-PCR and transient expression of TRPV4

Single-cell RT-PCR experiments were conducted as described previously, with slight modifications (Roudbaraki et al., 1999). In brief, after whole-cell recordings in single oviductal ciliated cells, the cell contents were aspirated under visual control. The pipette content (~8 μl) was ejected into an Eppendorf microtube with RNase inhibitor (20 U), and kept at –80°C until processed. Subsequently, one-step RT-PCR (QIAGEN) was performed using primers specific for a region of TRPV4 (GenBank/EMBL/DBJ accession no. 263523) highly conserved among vertebrates (from exon 7 to 12, nucleotides 1382–1918). Cloning of TRPV4 from human tracheal epithelial cells and transient expression in HEK-293 and HeLa cells were performed as described by Arniges et al. (2004).

### Immunodetection

Proteins from human TRPV4-transfected HEK-293 cells and hamster oviduct membranes were detected by Western blot technique using an affinity-purified polyclonal anti-human TRPV4 antibody (COOH-terminal residues CDGHQGGYPRKWRDAPL; dilution 1:500). Bands were visualized with HRP donkey anti-rabbit IgG (1:2,000) and chemiluminescence reagent (Supersignal West Femto; Pierce Chemical Co.). Confocal immunofluorescence using a TCS SP microscope (oil 40×, 1.25 NA; Leica) and software (Leica) were performed in HeLa-transfected cells. Cells were fixed and permeabilized as described previously (Bahamonde et al., 2003). Cells were incubated with the TRPV4 antibody (1:3,000) and secondary Alexa 594 goat anti-rabbit (1:1,000).

### Statistics

Data are expressed as the mean ± SEM. *t* test or ANOVA were performed with the SigmaPlot 5 or STATISTICA 6.0 programs. CBF data were analyzed after arcsin transformation. Tukey's test was used for post hoc comparison of means. The criterion for a significant difference was a final value of *P* < 0.05.

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