IP$_3$ sensitizes TRPV4 channel to the mechano-and osmotransducing messenger 5’-6’-epoxyeicosatrienoic acid

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Introduction

Clearance of mucus and pathogenic agents from lungs and the transport of gametes and embryos in the female reproductive organs are key functions of ciliated epithelia such as those present in the airways and the oviduct (Afzelius, 2004; for review see Salathe, 2007). The relevance of such processes is revealed by the association of defective mucociliary transport to human respiratory diseases (Houtmeyers et al., 1999) as well as to infertility (Afzelius, 2004). A critical factor in the maintenance of the appropriate velocity of mucociliary transport is the ciliary beat frequency (CBF; Puchelle et al., 1987). Although the regulation of CBF in vivo is largely under the control of chemical signals (for review see Salathe, 2007), both mechanical and chemical stimulation of ciliated cells are linked to the modulation of CBF by an intracellular Ca$^{2+}$ signal (Tamm, 1994; Lansley and Sanderson, 1999; for review see Salathe, 2007), although other mediators also participate (for review see Salathe, 2007). Increases in intracellular Ca$^{2+}$ concentration, at least in mammals, are almost always associated with increases in CBF (for review see Salathe, 2007). Mechanical stimulation in ciliated epithelia has been associated to extracellular Ca$^{2+}$ influx (Sanderson and Dirksen, 1986; Satir and Sleigh, 1990; Boitano et al., 1994), release of ATP (Okada et al., 2006; Winters et al., 2007), and inositol trisphosphate (IP$_3$)-mediated intracellular Ca$^{2+}$ release (Hansen et al., 1995; Felix et al., 1996; Homolya et al., 2000). Recently, the ability of hamster oviduct ciliated epithelial cells to adapt the CBF response to solutions of high viscosity (presumably by exerting a mechanical stimulation related to shear stress, viscous resistance to ciliary beat, or cell membrane fluctuations; Tuvia et al., 1997; Winters et al., 2007) has been shown to depend, at least in part, on a Ca$^{2+}$ signal generated by transient receptor potential vanilloid 4 (TRPV4) channel activation after exposure to high viscous solutions (Andrade et al., 2005).
The nonselective cation channel TRPV4 is a member of the vanilloid subfamily of transient receptor potential (TRP) channels (Montell, 2005). TRPV4 shows multiple modes of activation and regulatory sites, enabling it to respond to various stimuli, including osmotic cell swelling (Strotmann et al., 2000; Liedtke et al., 2000; Wissenbach et al., 2000; Arniges et al., 2004), mechanical stress (Gao et al., 2003; Suzuki et al., 2003; Liedtke et al., 2003; Andrade et al., 2005), heat (Guler et al., 2002), acidic pH (Suzuki et al., 2003), endogenous ligands (Watanabe et al., 2003), and both PKC-activating and nonactivating phorbol esters (Watanabe et al., 2002a; Xu et al., 2003). Besides, TRPV4 can be sensitized by coapplication of different stimuli (Gao et al., 2003; Alessandri-Haber et al., 2006; Grant et al., 2007). Osmotic (Vriens et al., 2004) and mechanical (Andrade et al., 2005) sensitivity of TRPV4 depends on phospholipase A$_2$ (PLA$_2$) activation and the subsequent production of the arachidonic acid (AA) metabolites, epoxyeicosatrienoic acids (EETs). Signaling pathways involving G proteins and/or PLC/IP$_3$ are also activated by osmotic cell swelling (Suzuki et al., 1990; Hoffmann and Dunham, 1995; Felix et al., 1996) and mechanical stimulation (Vandenburgh et al., 1993; Felix et al., 1996; Gudi et al., 1998). However, the contribution of these pathways to the generation of an osmotic or mechanically induced Ca$^{2+}$ signal by TRPV4 is unknown. Given that both extracellular ATP (Evans and Sanderson, 1999; Morales et al., 2000) and intracellular PLA$_2$ and PLC pathways (Hermoso et al., 2001; Barrera et al., 2004; Andrade et al., 2005) are involved in the regulation of CBF, we explored whether the PLC–IP$_3$ pathway might be involved in the response of the TRPV4 channel to high viscous solutions and hypotonic cell swelling. To do so, we measured TRPV4 activity in both hamster oviductal ciliated cells and TRPV4-expressing HeLa cells. We show here for the first time that IP$_3$, without being an agonist itself, sensitizes TRPV4 to EET but not to other TRPV4 physiological stimuli such as warm temperature, an effect that requires a functional IP$_3$ receptor (IP$_3$R).

**Results**

**Localization of TRPV4 in the hamster oviductal ciliated cells**

TRPV4 immunofluorescence in hamster oviduct was largely restricted to the cilia present in the columnar epithelium facing the lumen of oviduct sections (Fig. 1 A). Subcellular localization of TRPV4 was further evaluated in freshly dissociated oviductal cells. TRPV4 signal was clearly detected along the cilia, partially colocalized with the specific ciliary axoneme marker α-tubulin (Fig. 1 B, bottom right, yellow), although the strongest TRPV4 (green) and α-tubulin (red) signals were at the base (apical side of the cell) and the tip of cilia, respectively. A weak intracellular and basolateral membrane TRPV4 signal was also present in all cells analyzed. In controls where the primary antibody was omitted or in the presence of antigen-reabsorbed TRPV4 antibody, no signal was observed either at the cilia or apical location (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200712058/DC1).

**Relative contribution of PLA$_2$ and PLC pathways to high viscous loading- and osmotic-dependent stimulation of TRPV4**

We have previously demonstrated the activation of TRPV4 currents by high viscous loads in native oviductal ciliated cells and TRPV4-expressing HeLa cells (Andrade et al., 2005). We now approach the study of the intracellular signals that may contribute to the modulation of channel activity. Mechanical stimuli (including hypotonic stimuli) activate many different signaling pathways, among them PLA$_2$ and PLC (Vandenburgh et al., 1993; Hoffmann and Dunham, 1995). Moreover, CBF in hamster oviductal ciliated cells is under the control of both PLA$_2$ and PLC pathways (Hermoso et al., 2001; Barrera et al., 2004; Andrade et al., 2005). As an initial test for the hypothesis that various intracellular signaling pathways may be involved in the gating of TRPV4 by mechanical stimuli, we assessed whether inhibition of PLA$_2$ and PLC pathways blocked TRPV4 currents in response to high viscous solutions generated by adding 20% dextran to the control solution (Andrade et al., 2005) and osmotic (30% hypotonicity) stress, both stimuli related to different modalities of mechanical stress in the airways.

Under conditions favorable to measuring inward cationic currents (see Materials and methods), 20% dextran (Fig. 1 C) and 30% hypotonic (70% of normal osmolality) solutions (Fig. 1 D) evoked whole-cell TRPV4-like currents in isolated actively beating hamster oviduct cells. Inhibition of PLA$_2$ with 100 μM 4-bromophenacyl bromide (pBPB) or PLC with 1 μM U73122 totally blocked high viscosity-induced (20% dextran) TRPV4 current activation (Fig. 1 C), whereas hypotonicity (30%)-activated TRPV4 currents were completely inhibited only by pBPB (Fig. 1 D). Inhibition of PLA$_2$ with arachidonoyl trifluoromethyl ketone (AACOCF$_3$; 50 μM) also blocked dextran-induced TRPV4 currents (20% dextran: $-14.8 \pm 0.8$ pA/pF, $n = 7$; vs. dextran + AACOCF$_3$; $-1.9 \pm 0.6$ pA/pF, $n = 5$; $P < 0.05$). In the presence of U73122, significant hypotonicity-activated TRPV4 currents were recorded, although of smaller magnitude (Fig. 1 D). Mean normalized current responses obtained in all conditions described in Fig. 1 (C and D) are shown in Fig. 1 (E and F). The dramatic impact of the PLC inhibitor upon TRPV4 response to dextran-containing solutions contrasted with its modest effect upon hypotonic stimulation. This observation prompted us to analyze the signaling pathways up- and downstream of PLC activation. The participation of intracellular Ca$^{2+}$ stores in the activation of TRPV4 channel by 20% dextran solutions was discarded, as 1 μM thapsigargin, a blocker of the ER calcium pump, did not modify the TRPV4 response (Fig. 2 A). Transient cationic currents were observed after thapsigargin addition (not depicted) but disappeared within 5 min, the time at which cells were exposed to dextran solutions in the presence of thapsigargin (Fig. 2 A). High viscosity–induced currents were prevented in cells loaded with 500 μM GDPβ-s, which locks G proteins in their inactive state (Fig. 2 B), or treated with 100 μM of the P2 receptor antagonist suramin (Fig. 2 C). Mean normalized current responses obtained in the conditions described in Figs. 2 (A–C) are shown in Fig. 2 D. GDPβ-s also reduced the hypotonicity-induced currents to the same levels recorded in...
Consistent with the electrophysiological experiments, inhibition of PLC with U73122 did not modify the basal Ca\(^{2+}\) signal but prevented the oscillatory Ca\(^{2+}\) signal generated by 20% dextran in primary cultures of hamster oviductal ciliated cells (Fig. 3, A and B), representative single-cell Ca\(^{2+}\) signal is shown in Fig. 3 A, inset). U73122 also prevented the oscillatory Ca\(^{2+}\) signal generated by 30% hypotonic solutions (Fig. 3, E and F; representative single-cell Ca\(^{2+}\) signal in Fig. 3 E, inset) but maintained the initial peak (Fig. 3 F). The inactive isoform U73343 was without effect (Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200712058/DC1). The presence of 10 U/ml apyrase, an enzyme that rapidly hydrolysates nucleotide triphosphates to monophosphates, in the bathing solution mimicked the effect of PLC inhibition by U73122, which suggests a role for ATP release in the activation of the PLC–Ca\(^{2+}\) signaling (Fig. 3, C and G). A quantitative analysis of the Ca\(^{2+}\) signal, calculating the mean area under the curve as an indicator of the magnitude of the Ca\(^{2+}\) signal, is shown in Fig. 3 (D and H).
The presence of extracellular Ca$^{2+}$ (Fig. 3, A and E), where 76% (253/334) and 86% (69/80) of cells showed maintained oscillatory responses to 20% dextran and 30% hypotonic stimuli, respectively. The Ca$^{2+}$ peaks observed in Ca$^{2+}$-free solutions (Fig. 4, A and B) originated from intracellular stores, most likely IP$_3$-sensitive stores, as the signal disappeared in the presence of U73122 (Fig. 4 C). Under conditions where the PLC–IP$_3$ pathway was intact but ER Ca$^{2+}$ stores are depleted with thapsigargin, initial transient peaks were recorded in response to 20% dextran and 30% hypotonicity but the oscillatory pattern was lost (Fig. S3, C and D). Thus, in Ca$^{2+}$-containing solutions, the initial Ca$^{2+}$ peaks triggered by 20% dextran and 30% hypotonic solutions appear to involve both Ca$^{2+}$ entry and intracellular release, whereas maintained oscillations after the initial peak were mainly dependent on intracellular Ca$^{2+}$ stores.

In the presence of U73122 and apyrase, the response to 30% hypotonicity doubles the response to 20% dextran. Altogether, these observations suggested a new regulatory intracellular pathway, PLC-IP$_3$, participating in the channel gating by high viscosity solutions.

These experiments raised the question of whether TRPV4 also participates in the generation of the oscillatory Ca$^{2+}$ signals elicited by high-viscosity and hypotonic solutions. To address this point, we focused on the role of Ca$^{2+}$ entry in the maintenance of the oscillatory signals. Upon withdrawal of extracellular Ca$^{2+}$, 20% dextran (Fig. 4 A) or 30% hypotonic solutions (Fig. 4 B) generated a transient peak followed by oscillating Ca$^{2+}$ signals that ceased soon after the application of the stimuli only in 29% (23/77) and 9% (11/117) of cells, respectively. In the rest of the cells, no Ca$^{2+}$ signal was detected. These responses were very different from those obtained in the presence of extracellular Ca$^{2+}$ (Fig. 3, A and E), where 76% (253/334) and 86% (69/80) of cells showed maintained oscillatory responses to 20% dextran and 30% hypotonic stimuli, respectively. The Ca$^{2+}$ peaks observed in Ca$^{2+}$-free solutions (Fig. 4, A and B) originated from intracellular stores, most likely IP$_3$-sensitive stores, as the signal disappeared in the presence of U73122 (Fig. 4 C). Under conditions where the PLC–IP$_3$ pathway was intact but ER Ca$^{2+}$ stores are depleted with thapsigargin, initial transient peaks were recorded in response to 20% dextran and 30% hypotonicity but the oscillatory pattern was lost (Fig. S3, C and D). Thus, in Ca$^{2+}$-containing solutions, the initial Ca$^{2+}$ peaks triggered by 20% dextran and 30% hypotonic solutions appear to involve both Ca$^{2+}$ entry and intracellular release, whereas maintained oscillations after the initial peak were mainly dependent on intracellular Ca$^{2+}$ stores.
IP\textsubscript{3} sensitizes TRPV4 response to physical stimuli that did not reach the threshold level of PL\textsubscript{A}\textsubscript{2}–EET pathway activation

Based on the different effects of PL\textsubscript{A}\textsubscript{2} and PLC pathway inhibitors on TRPV4-like current and Ca\textsuperscript{2+} signal, we posited a signaling scenario in which the AA metabolites are the sole activators of TRPV4 in response to high viscous and hypotonic solutions, with IP\textsubscript{3} exerting a sensitizing effect on EET-induced TRPV4 currents; this is more evident under situations of low EET production. We hypothesized that the main difference between the two stimuli applied is the level of PL\textsubscript{A}\textsubscript{2} activation, being larger in response to hypotonic (30%) rather than high-viscosity (20% dextran) stimulation. Therefore, activation of TRPV4 in the former condition is less sensitive to inhibition of the PLC–IP\textsubscript{3} pathway. In addition, IP\textsubscript{3} alone should not induce significant TRPV4 activation but rather sensitize channel response to low EET concentrations. These two conditions were tested experimentally.

We first tested whether the level of PL\textsubscript{A}\textsubscript{2} activation is responsible for the different response seen under high viscous and hypotonic solutions. In the absence of a reliable method to directly test PL\textsubscript{A}\textsubscript{2} activity in hamster oviductal ciliated cells, we measured the Ca\textsuperscript{2+} signal and its dependence on PLC–IP\textsubscript{3} in ciliated cells under milder hypotonic stimuli (15%), aiming to elicit less PL\textsubscript{A}\textsubscript{2} activation. Fig. 5 (A and B) shows that, unlike the Ca\textsuperscript{2+} response to 30% hypotonic solutions (Fig. 3 F), the response to 15% hypotonicity is completely abolished by U73122. These results suggest that PLC–IP\textsubscript{3} pathway also becomes crucial to the generation of the Ca\textsuperscript{2+} signal under conditions of lower PL\textsubscript{A}\textsubscript{2} activation by milder hypotonic stimuli.

Second, TRPV4 currents were recorded in isolated ciliated cells dialyzed with pipette solutions containing 5\textsuperscript{H}H\textsubscript{11032},6\textsuperscript{H}H\textsubscript{11032}-EET at different concentrations (Fig. 5 C), obtaining an EC\textsubscript{50} of 3.2 ± 1.2 nM. TRPV4 channel response to 1 nM 5\textsuperscript{H}H\textsubscript{11032},6\textsuperscript{H}H\textsubscript{11032}-EET was greatly potentiated by the presence of 30 μM IP\textsubscript{3}, a concentration that elicits maximal activation of IP\textsubscript{3}R type 1 (IP\textsubscript{3}R1) and 3 (IP\textsubscript{3}R3; De Smet P. et al., 1999) in the pipette solution (Fig. 5 D), reaching maximal TRPV4 activation in hamster oviduct ciliated epithelial cells. These experiments confirmed that IP\textsubscript{3} does not seem to activate TRPV4 but may promote its activation in response to dextran and hypotonic solutions under conditions where PLC is inhibited (Fig. 2, E and F) or in cells loaded with 1 nM 5\textsuperscript{H}H\textsubscript{11032},6\textsuperscript{H}H\textsubscript{11032}-EET (Fig. 5 D).

IP\textsubscript{3}R mediates IP\textsubscript{3}-dependent sensitization of TRPV4 to 5\textsuperscript{H},6\textsuperscript{H}-EET

Next, we further investigated the mechanism of IP\textsubscript{3}-mediated sensitization. A previous study has localized IP\textsubscript{3}R3 (not IP\textsubscript{3}R1) to the plasma membrane of hamster oviduct ciliated cells and suggested its participation in Ca\textsuperscript{2+} influx (Barrera et al., 2004). To test whether IP\textsubscript{3}-mediated sensitization was a general mechanism rather than circumscribed to ciliated cells, we expressed IP\textsubscript{3} sensitizes TRPV4 response to physical stimuli that did not reach the threshold level of PL\textsubscript{A}\textsubscript{2}–EET pathway activation.
human TRPV4 in HeLa cells that endogenously expressed IP₃R1 and IP₃R3 (Tovey et al., 2001).

Dialysis, through the patch pipette, of HeLa cells expressing human TRPV4 with 1 nM 5ʹ,6ʹ-EET and 30 μM IP₃ resulted in an increase in current that reached a maximum within 3–5 min (Fig. 6 A). The current-voltage relationship of the corresponding TRPV4 currents is shown in Fig. 6 D. Dialysis with EET and/or IP₃ alone elicited no significant TRPV4-like currents (Fig. 6 D). The cationic currents shown in Fig. 6 (A and D) illustrate that the IP₃-mediated sensitization observed in hamster ciliated cells is reconstituted in HeLa cells expressing human TRPV4. Mean normalized currents are shown in Fig. 6 H. Increasing 5ʹ,6ʹ-EET concentration to 100 nM in the presence of 30 μM IP₃ augmented TRPV4 current amplitude (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200712058/DC1), although it did not reach statistical significance compared with 1 nM 5ʹ,6ʹ-EET + 30 μM IP₃ (Fig. 6 H) or 100 nM 5ʹ,6ʹ-EET alone (Fig. S4). No sensitization was observed in HeLa cells transfected with rat IP₃R3 (Fig. 6, C and F) or EGFP alone (Fig. 6 G). Coexpression of TRPV4 and IP₃R3 (Fig. 6, B, E, and I) tripled IP₃ potentiation as compared with TRPV4 expression alone, an effect that was completely inhibited in the presence of 1 μM of the IP₃R inhibitor xestospongin C. Neither EET (1 nM) nor IP₃ alone activated TRPV4 currents in HeLa cells expressing any combination of EGFP, TRPV4, or IP₃R3 (P > 0.05).

As an independent test for the role of PLC–IP₃ signaling in the sensitization of TRPV4, we evaluated the TRPV4-mediated Ca²⁺ signals obtained in response to 30% hypotonic and 20% dextran solutions in the presence or absence of extracellular ATP in HeLa cells expressing C-terminal YFP-tagged TRPV4 and IP₃R3. Cationic currents recorded from TRPV4-YFP–transfected cells presented the same electrophysiological properties (not depicted) than those recorded from TRPV4-transfected cells (Fig. 6). As shown in Fig. 7 A, cells were first challenged with a 30% hypotonic solution for 5 min and then challenged with a hypotonic solution containing 1 μM ATP. The presence of extracellular ATP significantly enhanced the hypotonicity-induced Ca²⁺ signal. In the absence of extracellular ATP, the second hypotonic shock generated a smaller Ca²⁺ signal (Fig. 7 D). Neither hypotonicity-induced Ca²⁺ signal or its enhancement by ATP were observed in HeLa cells overexpressing IP₃R3 alone (Fig. 7 F). The addition of 1 μM ATP alone was insufficient to elicit a Ca²⁺ signal, although cells responded to 100 μM ATP (Fig. 7 C). The presence of low concentrations of extracellular ATP was also essential to record Ca²⁺ signals in response to 20% dextran solutions in HeLa cells expressing TRPV4 and IP₃R3 (Fig. 7 B). In the absence of ATP, no Ca²⁺ signal was recorded in response to 20% dextran solutions (Fig. 7 E), although the cell responded to the TRPV4 agonist 4α-phorbol 12,13-didecanoate (4α-PDD; 10 μM). Similar results were obtained in CHO, HEK, and COS cells transfected with human TRPV4 (n > 200; unpublished data). HeLa cells expressing IP₃R3 showed no response to dextran solutions containing 1 μM ATP (Fig. 7 G). Mean increases in the Ca²⁺ signal obtained in the conditions described in Fig. 7 (A–G) are shown in Fig. 7 (H–J). Altogether, these data suggest that ATP–PLC–IP₃
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et al., 2002b). Increasing the bathing solution temperature from 22 to 35°C transiently activated TRPV4 channels in TRPV4-IP3R3-expressing HeLa cells (Fig. 9A). However, unlike the EET-induced TRPV4 activation, warm temperature response was unaffected by IP3 (Fig. 9B). Current-voltage relations of peak whole-cell cationic currents were recorded from different HeLa cells transfected with TRPV4 (D) or TRPV4 and IP3R3 (E) and dialyzed with either 1 nM 5,6′-EET, 30 μM IP3, or EET+IP3. (F) Current densities at −100 mV in HeLa cells transfected with IP3R3 and dialyzed with 1 nM 5,6′-EET (n = 4), 30 μM IP3 (n = 5), and EET+IP3 (n = 4). (G) EGFP transfected cells dialyzed with 1 nM 5,6′-EET (n = 3), 30 μM IP3 (n = 4), and EET+IP3 (n = 4). (H) Current densities at −100 mV in HeLa cells expressing TRPV4: control (n = 4); 1 nM 5,6′-EET (n = 7); 30 μM IP3 (n = 8); and EET + IP3 (n = 8). (I) Current densities at −100 mV in HeLa cells expressing TRPV4 and IP3R3: control (n = 6); 1 nM 5,6′-EET (n = 10); 30 μM IP3 (n = 11); EET + IP3 (n = 8); and EET + IP3 + 1 μM xestospongin C (n = 4). Data are expressed as the mean ± SEM; **, P < 0.001 (one-way ANOVA and Bonferroni post hoc).

Figure 6. IP3R involvement in IP3-dependent sensitization of TRPV4 to 5,6′-EET. Time course of whole-cell cationic currents at −100 (●) and 100 mV (○) in HeLa cells transfected with human TRPV4 (A), TRPV4 and rat IP3R3 (B), or IP3R3 (C) and dialyzed with 1 nM 5,6′-EET and 30 μM IP3. At the time indicated by the bars, the NaCl bathing solution was replaced by N-methyl-D-glucamine chloride solution (NMDGCl) in A and B. Current-voltage relations of peak whole-cell cationic currents were recorded from different HeLa cells transfected with TRPV4 (D) or TRPV4 and IP3R3 (E) and dialyzed with either 1 nM 5,6′-EET, 30 μM IP3, or EET+IP3. (F) Current densities at −100 mV in HeLa cells transfected with IP3R3 and dialyzed with 1 nM 5,6′-EET (n = 4), 30 μM IP3 (n = 5), and EET+IP3 (n = 4). (G) EGFP transfected cells dialyzed with 1 nM 5,6′-EET (n = 3), 30 μM IP3 (n = 4), and EET+IP3 (n = 4). (H) Current densities at −100 mV in HeLa cells expressing TRPV4: control (n = 4); 1 nM 5,6′-EET (n = 7); 30 μM IP3 (n = 8); and EET + IP3 (n = 8). (I) Current densities at −100 mV in HeLa cells expressing TRPV4 and IP3R3: control (n = 6); 1 nM 5,6′-EET (n = 10); 30 μM IP3 (n = 11); EET + IP3 (n = 8); and EET + IP3 + 1 μM xestospongin C (n = 4). Data are expressed as the mean ± SEM; **, P < 0.001 (one-way ANOVA and Bonferroni post hoc).

Discussion

In ciliated epithelia, Ca2+ plays a crucial role in CBF regulation and, consequently, the transport of mucus and trapped particles (Satir and Sleigh, 1990; Salathe, 2007). Ciliated cells respond to mechanical stimulation (an important aspect in mucociliary transport and defense) with increases in intracellular Ca2+ and CBF (Sanderson and Dirksen, 1986), a process in which mucus viscosity has been considered as a physiological factor initiating or modulating the response (Spungin and Silberberg, 1984; Winters et al., 2007). Therefore, understanding the regulation of the Ca2+ influx pathway and its implication in the generation of the Ca2+ signal is essential to comprehend ciliated epithelia’s response to mechanical stimulation in the context of both physiological readout.

IP3 does not sensitize TRPV4 to warm temperature

We also evaluated whether IP3 sensitizes the response of TRPV4 to warm temperature (Guler et al., 2002b). Increasing the bathing solution temperature from 22 to 35°C transiently activated TRPV4 channels in TRPV4-IP3R3-expressing HeLa cells (Fig. 9A). However, unlike the EET-induced TRPV4 activation, warm temperature response was unaffected by IP3 (Fig. 9B). Current-voltage traces and mean responses to warm temperatures in the presence or absence of 30 μM IP3 in the pipette solution are shown in Fig. 9 (C and D).
and pathological conditions (Houtmeyers et al., 1999; Afzelius, 2004). Mechanically generated Ca\(^{2+}\) signals have been classically attributed to the activation of mechanosensitive Ca\(^{2+}\) entry pathways at the plasma membrane or Ca\(^{2+}\) release from IP\(_3\)-sensitive intracellular stores (McCarty and O’Neil, 1992; Sachs and Morris, 1998). Among the possible candidates to mediate Ca\(^{2+}\) entry, TRP channels are well placed, as many of them respond to osmotic and/or mechanical stimuli (Christensen and Corey, 2007). Several members of the TRP family of channels have been found in epithelial tissues, although to date, only TRPV4, TRPP1-2, TRPA1, TRPN1, and TRPML3 have been identified in ciliated epithelial cells, including inner ear hair cells (Andrade et al., 2007).

Different pieces of evidence have pointed to TRPV4 as the Ca\(^{2+}\) entry channel in response to high viscous and hypotonic solutions in native hamster oviductal ciliated epithelial cells (Andrade et al., 2005; Teilmann et al., 2005): (1) TRPV4 mRNA and protein have been identified in oviductal ciliated cells; (2) electrophysiological characterization of high-viscosity- and hypotonicity-induced cationic currents in ciliated cells coincides with the features of cationic currents induced by the TRPV4-specific agonist 4\(\pi\)H9251-PDD; (3) functional inhibition of the high viscosity–induced cationic current with an antibody against TRPV4 in oviductal ciliated cells; and (4) high-viscosity solutions evoked cation currents and Ca\(^{2+}\) signals in TRPV4-expressing HeLa cells but not in cells transfected with vector alone. The present study adds new evidence supporting the participation of TRPV4 in the response of ciliated cells to high-viscosity and hypotonic solutions: the presence of TRPV4 at the base of the cilia, where oscillatory Ca\(^{2+}\) signals are needed to modulate CBF (Tamm, 1994; Evans and Sanderson, 1999; Lansley and Sanderson, 1999), and the convergence of signaling

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**Figure 7.** TRPV4 response to high viscous and hypotonic solutions is potentiated by ATP. Representative intracellular Ca\(^{2+}\) signals (Δ ratio, 340/380) obtained from cells transfected with the indicated constructs and exposed to the conditions shown in the bars. (H–J) Mean increases in 340/380 signal under the experimental conditions shown in A–G. Results are mean ± SEM of multiple cells recorded from four independent experiments for each condition. Data are expressed as the mean ± SEM. Significant differences (P < 0.05) between groups were marked with a single (vs. control) or double asterisk (hypo + ATP vs. hypo; one way ANOVA and Bonferroni post hoc).
pathways in the TRPV4 activation by high viscosity and hypertonic solutions in both native ciliated cells and in cells heterologously expressing the channel. All together, these observations are consistent with the role of TRPV4 in the transduction of mechanical stimulation in ciliated epithelial cells (Andrade et al., 2005). Ciliated epithelia of the oviduct also express TRPP1-2 (Teilmann et al., 2005), although their functional significance is still unresolved.

We now demonstrate for the first time that: (1) PLC–IP₃ signaling participates in TRPV4 activation by high-viscosity solutions in hamster oviductal ciliated cells downstream to the activation of P2 receptors after mechanically induced ATP release (Felix et al., 1996; Okada et al., 2006; Winters et al., 2007); under our experimental conditions, the suramin-sensitive receptor implicated is most likely of the P2Y₂ type, which has been associated to the mechanosensitivity of ciliated epithelial cells (Winters et al., 2007) and is present in hamster oviductal ciliated cells (Morales et al., 2000); (2) that IP₃ alone is able to compensate the inhibitory effect of U73122; (3) that the effect of IP₃ requires a functional IP₃R (as the sensitizing effect is
inhibited by xestospongin C) although it does not require the release of Ca\(^{2+}\) via IP\(_3\)R, as the response is maintained in cells in which ER was calcium-depleted using thapsigargin; and (4) the possibility that IP\(_3\)-mediated potentiation of TRPV4 response to dextran solutions involves positive feedback via a Ca\(^{2+}\)-calmodulin–dependent mechanism (Strotmann et al., 2003) is unlikely, as TRPV4 currents were recorded in the absence of extracellular and intracellular Ca\(^{2+}\) (including 5 mM EGTA) and in the presence of thapsigargin.

Both hypotonic and mechanical stimulation activates PLC and/or PLA\(_2\) in different cell types (Lehtonen and Kinnunen, 1995; Pedersen et al., 2000; Moore et al., 2002; Zholos et al., 2005), although, to date, only the latter has been implicated in TRPV4 regulation. Activation of both signaling pathways has been associated with direct sensing by the phospholipid bilayer of physical stimuli and activation of membrane-bound G proteins in the case of PLC (Gudi et al., 1998, 2003) or direct activation of PLA\(_2\) (Lehtonen and Kinnunen, 1995; Pedersen et al., 2000). Moreover, crosstalk between PLC and PLA\(_2\) has been demonstrated in several cell types (Vandenburgh et al., 1993). Activation of TRPV4 under hypotonic (Vriens et al., 2004) and high viscosity conditions (Andrade et al., 2005) depends on the activity of PLA\(_2\) and appears to be ultimately related to the production of 5,6\'-EET via the metabolism of AA by P450 enzymes. Thus, 5,6\'-EET is the only physiological, diffusible molecule known to directly activate TRPV4 (Watanabe et al., 2003). Other TRPV4 stimuli such as the viscosity of the 20% dextran solution and the synthetic 4a-PDD are independent of 5,6\'-EET production (Vriens et al., 2004).

The impact of the ATP–PLC–IP\(_3\) pathway on TRPV4 activity depends on the stimuli used, being more relevant in the case of channel activation by 20% dextran solutions than in the case of 30% hypotonic solutions, probably reflecting a higher level of PLA\(_2\) activity in the latter. However, using a milder hypotonic stimuli (15%) turned the response fully PLC dependent. IP\(_3\) also potentiated TRPV4 response to low EET concentrations measured by whole-cell patch clamp of both active PLA\(_2\) and PLA\(_2\)–AA–EET signaling is essential for the activation of TRPV4 by high viscous and hypotonic solutions that do not reach a threshold level of PLA\(_2\) activity. Our data also addresses the impact of Ca\(^{2+}\) entry upon agonist-induced Ca\(^{2+}\) oscillations (Yule and Gallagher, 1988; Shuttleworth, 1999). The dependency on Ca\(^{2+}\) entry for continued oscillations has been interpreted in terms of the Ca\(^{2+}\) dependency of the IP\(_3\)R (Shuttleworth, 1999). Under conditions of low activation of the PLC–IP\(_3\) pathway (usually associated to oscillatory Ca\(^{2+}\) signals), IP\(_3\) will bind to IP\(_3\)R and release little or no stored Ca\(^{2+}\), a response that is magnified by the sensitizing effect of Ca\(^{2+}\) entry via plasma membrane channels situated in close proximity to the IP\(_3\). Using this model, we propose that the PLC–IP\(_3\) pathway is required for PLA\(_2\)–dependent TRPV4 activation by dextran solutions and that both active TRPV4 and the PLC–IP\(_3\) pathway are needed to maintain the oscillatory Ca\(^{2+}\) signal. In the case of 30% hypotonic stimuli, TRPV4 activation is largely independent of PLC–IP\(_3\) pathway but, again, both active TRPV4 and the PLC–IP\(_3\) pathway are needed to maintain oscillations. In this sense, it is worth mentioning that, although the basic features of the TRPV4 response in native epithelia are reproduced in cells heterologously expressing the channel, the overall Ca\(^{2+}\) signal recorded in response to 20% dextran and 30% hypotonic solutions was not fully reproduced in the cell expression systems. Ciliated epithelial cells responded with oscillatory Ca\(^{2+}\) signals to both stimuli (Fig. 3), whereas HeLa cells expressing TRPV4 responded with single, transient Ca\(^{2+}\) increases (Fig. 7). Occasionally, additional peaks were observed in HeLa cells (Fig. 7 B). Another difference between the response of ciliated epithelia and HeLa cells expressing TRPV4 is the impact of the ATP–PLC–IP\(_3\) pathway on TRPV4 activation by 20% dextran solutions. Although ciliated cells responded to dextran solutions in the absence of added ATP, HeLa cells required the presence of 1 μM ATP to respond to dextran solutions. The difference may reflect a higher efficiency of ciliated cells to release ATP in response to mechanical/osmotic stimuli, a higher sensitivity of the ATP–PLC–IP\(_3\) pathway to extracellular ATP, or more efficient coupling between the ATP–PLC–IP\(_3\) and PLA\(_2\)–AA–EET pathways to activate TRPV4. At present, we cannot discriminate between these three possibilities, and this remains an interesting issue for future studies.

Conclusions

We have delineated a novel regulatory mechanism through which IP\(_3\) via its receptor, potentiates TRPV4 sensitivity to the mechano- and osmotransducing messenger 5,6\'-EET but not to thermal stimulation. However, at present it is not known whether the association between TRPV4 and IP\(_3\)R demonstrated by the coimmunoprecipitation studies is required to support the potentiating effect of IP\(_3\). IP\(_3\)Rs are, themselves, capable of mediating plasma membrane Ca\(^{2+}\) entry (Dellis et al., 2006) or interacting with and modulating the TRPC and TRPP channels (Boulay et al., 1999; Kiseliov et al., 2005; Li et al., 2005), although no description of such mechanisms exists for the subfamily of TRPV channels (Clapham, 2003). Thus, IP\(_3\)R, without being a channel itself or a being direct activator of plasma membrane ion channels, modulates Ca\(^{2+}\) influx via TRPV4. This mechanism is another example of the complexity of TRP channel gating, most likely reflecting the physiologically relevant convergence of different signaling pathways into channel gating. In conclusion, the functional coupling between IP\(_3\)R and TRPV4 ensures channel gating under conditions of mechanical stimulation that do not reach a threshold level of PLA\(_2\)–EET pathway activation.

Materials and methods

Chemicals and solutions

All chemicals were obtained from Sigma-Aldrich except dextran T.500 (500,000 D; GE Healthcare), fura-2-AM (Invitrogen), AAOCOF\(_2\), and pBPB (EMD). Isotonic bathing solutions used for imaging experiments contained 140 mM NaCl, 5 mM KCl, 1.2 mM Ca\(_{2+}\), 0.5 mM MgCl\(_2\), 5 mM glucose, and 10 mM Hepes, pH 7.4, at 300 mosmol/liter. For electrophysiology bathing solutions, Ca\(_{2+}\) was removed and 1 mM MgCl\(_2\) and 1 mM EGTA were added. 30% and 15% hypotonic solutions were obtained by removing 40 mM and 20 mM NaCl, respectively (255 and 220 mosmol/liter). The viscosity of the 20% dextran solution was increased by adding
dextran T-500, which does not change the osmolality (300 mosmol/liter). All inhibitors were added 5 min before stimulation.

Cells
Primary cultures and single ciliated cells were obtained and maintained as described previously (Lock and Valverde, 2000; Hermoso et al., 2001; Andrade et al., 2003). Animals were maintained and experiments were performed according to the guidelines issued by the Institutional Ethics Committees of the Institut Municipal d’Investigació Mèdica of the Universitat Pompeu Fabra. All experiments were performed only in beating ciliated cells. Hela cells were transfected with different combinations of the following cDNAs as described previously (Arniges et al., 2000, 2004, 2006; Andrade et al., 2005) was used at 6.4 μg/ml. A commercial anti-α-tubulin (Sigma-Aldrich) was diluted to 1:500. For immunodetection, we used goat anti-rabbit IgG Alexa 488 (Invitrogen) and goat anti-mouse IgG Alexa 555 (Invitrogen) diluted 1:750 in the same solution used with the primary antibodies. Samples were counterstained with 1 μg/ml TO-PRO-3 in PBS for nuclear localization. Images were taken at RT with an inverted confocal microscope (SP2; Leica) using an HCX Pl APO 63 ×  1.32 NA oil Ph3 confocal scanning objective (Leica), LCS confocal software (Leica) and argon lasers. Original images were not further processed except for adjustments of brightness, contrast, and color balance.

Measurement of intracellular Ca2+
Cytosolic Ca2+ signal was determined at 30–37°C in cells loaded with 4.5 μM fura-2AM as described previously (Fernandez-Fernandez et al., 2002; Arniges et al., 2004, 2006). Cytosolic Ca2+ increases are presented as the increment in the ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm relative to the baseline.

Electrophysiology
Ionic currents were recorded in the whole-cell patch-clamp mode (Fernandez-Fernandez et al., 2002). Patch pipettes were filled with a solution containing 140 mM N-methyl-D-glucamine chloride, 1 mM MgCl2, 5 mM EGTA, 10 mM Hepes, 4 mM ATP, and 0.1 mM GTP (300 mosmolos/liter, pH 7.3). Occasionally, pipette solutions contained different concentrations of 5, 6-EET and IP3 as shown in the corresponding figures. 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 10 kHz and low-pass filtered at 1 kHz. Experiments were performed at RT (22–26°C). In case of TRPV4 activation by heat, temperature of the bathing solution was changed within 30 s.

Immunoprecipitation assay, SDS-PAGE, and Western blotting
TRPV4-YFP and IP3R3 receptor transfected and nontransfected Hela cells (48 h) were washed twice with cold PBS and detached using a cell scraper. Whole-cell extracts were obtained and protein concentration was assessed using the Bradford method. 200 μg of total protein samples from transfected and nontransfected Hela cells were incubated overnight at 4°C, gently mixed with 4 μl of anti-GFP polyclonal antibody (rabbit; Clontech Laboratories, Inc., or) and 2,700 μg of total protein and 27 μl of anti-IP3R3 antibody (mouse; BD Biosciences) for the reverse coimmunoprecipitation. After that, 15 μg of protein G was added to the samples and mixtures were incubated for 2 h at room temperature. Protein G immunocomplexes were collected by centrifugation, washed four times with PBS, and resuspended in Laemmli sample buffer with 5% β-mercaptoethanol. Samples were boiled for 6 min at 100°C and centrifuged for 10 min at 13,000 rpm to remove protein G. Supernatants were collected, boiled again for 3 min at 100°C, electrophoresed in 8% Tris·HCl polyacrylamide gels, and transferred to nitrocellulose membranes using a dry blotting system (iBlot; Invitrogen). Membranes were blocked overnight at 4°C in Tris Base solution 1×–0.1% Tween 20 containing either 5% skim milk or 3% BSA. Membranes were washed again and subjected to chemiluminescence analysis using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and detected on ECL films (GE Healthcare). Primary antibodies used for Western blotting were anti-GFP for TRPV4 detection (1:1,100, rabbit; Clontech Laboratories, Inc.), anti-IP3R3 (1:3,000, mouse; BD Biosciences), and anti-IP3R1 (1:1,200, rabbit; Millipore). Mouse and rabbit secondary antibodies (GE Healthcare) from a sheep and donkey source, respectively, were used at 1:2,000.

Statistics
Data are expressed as the mean ± SEM. Student’s t test or analysis of variance (ANOVA) were performed with the SigmaPlot (Systat Software, Inc.) and SPSS (SPSS, Inc.) programs. Bonferroni’s test was used for post hoc comparison of means.

Online supplemental material
Fig. S1 shows immunofluorescence images of an antigen-preabsorbed TRPV4 antibody. Fig. S2 shows the effect of GDPβS, U73122, IP3, and thapsigargin on cationic currents recorded from hamster oviducal ciliated cells stimulated with 20% dextran or 30% hypotonic solutions. Fig. S3 shows calcium imaging data obtained from hamster oviducal ciliated cells in response to 20% dextran or 30% hypotonic solutions in the presence of U73343 or thapsigargin. Fig. S4 shows potentiation of the TRPV4 current by IP3 at different EET concentrations in TRPV4-expressing Hela cells. Fig. S5 shows reverse coimmunoprecipitation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200712058/DC1.

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