Mechanical and osmotic sensitivity of the transient receptor potential vanilloid 4 (TRPV4) channel depends on phospholipase A$_2$ (PLA$_2$) activation and the subsequent production of the arachidonic acid metabolites, epoxyeicosatrienoic acid (EET). We show that both high viscous loading and hypotonicity stimuli in native ciliated epithelial cells use PLA$_2$–EET as the primary pathway to activate TRPV4. Under conditions of low PLA$_2$ activation, both also use extracellular ATP-mediated activation of phospholipase C (PLC)–inositol trisphosphate (IP$_3$) signaling to support TRPV4 gating. IP$_3$, without being an agonist itself, sensitizes TRPV4 to EET in epithelial ciliated cells and cells heterologously expressing TRPV4, an effect inhibited by the IP$_3$ receptor antagonist xestospongin C. Coimmunoprecipitation assays indicated a physical interaction between TRPV4 and IP$_3$ receptor 3. Collectively, our study suggests a functional coupling between plasma membrane TRPV4 channels and intracellular store Ca$^{2+}$ channels required to initiate and maintain the oscillatory Ca$^{2+}$ signal triggered by high viscosity and hypotonic stimuli that do not reach a threshold level of PLA$_2$ activation.

**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), mechanical stimulation has also been proposed as a participant in the physiological regulation of CBF (Sanderson and Dirksen, 1986), with the highly viscous mucus being a relevant factor in the generation of the mechanical stimuli (Spungin and Silberberg, 1984; Satir and Sleigh, 1990). 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 with 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 A2 (PLA2) activation and the subsequent production of the arachidonic acid (AA) metabolites, epoxyeicosatrienoic acids (EETs). Signaling pathways involving G proteins and/or PLC/IP3 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 Ca2+ signal by TRPV4 is unknown. Given that both extracellular ATP (Evans and Sanderson, 1999; Morales et al., 2000) and intracellular PLA2 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–IP3 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 IP3, 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 IP3 receptor (IP3R).

**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 PLA2 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 PLA2 and PLC (Vandenburgh et al., 1993; Hoffmann and Dunham, 1995). Moreover, CBF in hamster oviductal ciliated cells is under the control of both PLA2 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 PLA2 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 PLA2 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 PLA2 with arachidonyl trifluoromethyl ketone (AACOCF3; 50 μM) also blocked dextran-induced TRPV4 currents (20% dextran: −14.8 ± 0.8 pA/pF, n = 7; vs. dextran + AACOCF3: −1.9 ± 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 Ca2+ 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 Fig. 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 hydrolyses 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–IP\(_3\) pathway. Dialysis of cells with a pipette solution containing 30 μM IP\(_3\) restored TRPV4 activation by dextran solutions in the presence of U73122 (PLC inhibitor) except when both U73122 and pBPB (PLA\(_2\) inhibitor) were used (Fig. 2, E and F). Similarly, the presence of IP\(_3\) in the pipette solution also restored full TRPV4 activation by 30% hypotonic solutions (Fig. S2 A). The combination of pipette solutions containing IP\(_3\) and cell stimulation with 20% dextran solutions showed a modest but significant potentiation of the response, which was not modified by treatment with thapsigargin (Fig. S2 B), therefore confirming the little impact of intracellular stores to the IP\(_3\)-mediated sensitization of TRPV4 response to highly viscous loads.
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.

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.
IP₃ sensitizes TRPV4 response to physical stimuli that did not reach the threshold level of PLA₂-EET pathway activation

Based on the different effects of PLA₂ and PLC pathway inhibitors on TRPV4-like current and Ca²⁺ signal, we postulated a signaling scenario in which the AA metabolites are the sole activators of TRPV4 in response to high viscous and hypotonic solutions, with IP₃ 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 PLA₂ 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–IP₃ pathway. In addition, IP₃ 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 PLA₂ activation is responsible for the different response seen under high viscous and hypotonic solutions. In the absence of a reliable method to directly test PLA₂ activity in hamster oviductal ciliated cells, we measured the Ca²⁺ signal and its dependence on PLC–IP₃ in ciliated cells under milder hypotonic stimuli (15%), aiming to elicit less PLA₂ activation.  Fig. 5 (A and B) shows that, unlike the Ca²⁺ response to 30% hypotonic solutions (Fig. 3 F), the response to 15% hypotonicity is completely abolished by U73122. These results suggest that PLC–IP₃ pathway also becomes crucial to the generation of the Ca²⁺ signal under conditions of lower PLA₂ activation by milder hypotonic stimuli.

Second, TRPV4 currents were recorded in isolated ciliated cells dialyzed with pipette solutions containing 5,6-EET at different concentrations (Fig. 5 C), obtaining an EC₅₀ of 3.2 ± 1.2 nM. TRPV4 channel response to 1 nM 5,6-EET was greatly potentiated by the presence of 30 μM IP₃ (Fig. 5 D), reaching maximal TRPV4 activation in hamster oviduct ciliated epithelial cells. These experiments confirmed that IP₃ 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 5,6-EET (Fig. 5 D).

IP₃R mediates IP₃-dependent sensitization of TRPV4 to 5,6-EET

Next, we further investigated the mechanism of IP₃-mediated sensitization. A previous study has localized IP₃R type 1 (IP₃R1) and 3 (IP₃R3; 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 IP₃ 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 5,6-EET (Fig. 5 D).

Figure 3. Effect of PLA₂ and PLC inhibitors on intracellular Ca²⁺ signals. Representative intracellular Ca²⁺ signals (Δ ratio, 340/380) obtained from different primary cultures of hamster oviductal ciliated cells stimulated with: (A) 20% dextran solution (n = 21; inset shows a recording obtained from a single cell); (B) dextran + 2 μM U73122 (n = 22); and (C) dextran + apyrase (10 U/ml; n = 19). (D) Mean [Ca²⁺] increases (Δ area under the curve) in response to 20% dextran. Intracellular Ca²⁺ signals obtained from (E) 30% hypotonic solution (n = 29; inset shows a recording obtained from a single cell); (F) hypotonicity + 2 μM U73122 (n = 17); and (G) hypotonicity + apyrase (10 U/ml; n = 29). (H) Mean [Ca²⁺] increases (Δ area under the curve) in response to hypotonicity. Data are expressed as the mean ± SEM; *, P < 0.05 for dextran and hypotonicity versus the inhibitors (one way ANOVA and Bonferroni post hoc).

Figure 4. Effect of removing extracellular Ca²⁺ on intracellular Ca²⁺ signals. (A) Representative intracellular Ca²⁺ signals obtained from primary cultures of hamster oviductal ciliated cells bathed in Ca²⁺-free solutions and stimulated with 20% dextran (n = 32), 30% hypotonicity (B; n = 41) and 30% hypotonicity in the presence of 2 μM U73122 (C; n = 25).
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 5',6'-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 5',6'-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 5',6'-EET + 30 μM IP₃ (Fig. 6 H) or 100 nM 5',6'-EET alone (Fig. S4). No sensitization was observed in HeLa cells transfected with rat IP₃ alone (Fig. 6 H) or IP₃ alone elicited no significant TRPV4-like currents (Figs. 5 A and B). No sensitization was observed in HeLa cells transfected with rat IP₃ alone (Fig. 6 H) or IP₃ alone elicited no significant TRPV4-like currents (Figs. 5 A and B). No sensitization was observed in HeLa cells transfected with rat IP₃ alone (Fig. 6 H) or IP₃ alone elicited no significant TRPV4-like currents (Figs. 5 A and B). No sensitization was observed in HeLa cells transfected with rat IP₃ alone (Fig. 6 H) or IP₃ alone elicited no significant TRPV4-like currents (Figs. 5 A and B). No sensitization was observed in HeLa cells transfected with rat IP₃ alone (Fig. 6 H) or IP₃ alone elicited no significant TRPV4-like currents (Figs. 5 A and B). No sensitization was observed in HeLa cells transfected with rat IP₃ alone (Fig. 6 H) or IP₃ alone elicited no significant TRPV4-like currents (Figs. 5 A and B). 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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
et al., 2002b). Increasing the bathing solution temperature from 22 to 35 °C transiently activated TRPV4 channels in TRPV4-IP3R3–expressing HeLa cells (Fig. 9 A). However, unlike the EET-induced TRPV4 activation, warm temperature response was unaffected by IP3 (Fig. 9 B). 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).

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 and pathophysiological conditions.

IP3 does not sensitize TRPV4 to warm temperature

We also evaluated whether IP3 sensitizes the response of TRPV4 to warm temperature (Guler et al., 2002; Watanabe et al., 2002b). Increasing the bathing solution temperature from 22 to 35°C transiently activated TRPV4 channels in TRPV4-IP3R3-expressing HeLa cells (Fig. 9 A). However, unlike the EET-induced TRPV4 activation, warm temperature response was unaffected by IP3 (Fig. 9 B). 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).

Figure 7. 
TRPV4 response to high viscous and hypotonic solutions is potentiated by ATP.

Representative intracellular Ca\(^{2+}\) signals (∆ 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) and or double asterisk (hypo + ATP vs hypo; one way ANOVA and Bonferroni post hoc).
pathways in the TRPV4 activation by high viscosity and hypotonic 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 temperature 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 native ciliated epithelia and cells heterologously expressing TRPV4, which is consistent with the observation that convergence of ATP–PLC–IP$_3$ and PLA$_{2}$–AA–EET pathways is essential for the activation of TRPV4 by high viscous and hypotonic solutions that do not reach a threshold level of PLA$_{2}$ activation.

Our data also addresses the impact of Ca$^{2+}$ entry upon agonist-induced Ca$^{2+}$ oscillations (Yule and Gallacher, 1988; Shuttleworth, 1999). The dependency on Ca$^{2+}$ entry for continued oscillations has been interpreted in terms of the Ca$^{2+}$ dependence 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$_{3}$R. 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 (50,000 D; GE Healthcare), fura2-AM (Invitrogen), AACOCF$_3$, and pBPB (EMD). Isotonic bathing solutions used for imaging experiments contained 140 mM NaCl, 5 mM KCl, 1.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5 mM glucose, and 10 mM Hepes, pH 7.4, at 300 mosmol/liter. For electrophysiology bathing solutions, CaCl$_2$ 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
incubated overnight at 4°C, gently mixed with 4 μl of anti-rGFP polyclonal antibody (rabbit; Clontech Laboratories, Inc.) or 2,700 μg of total protein and 27 μl of anti-IP3R 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-rGFP for TRPV4 detection (1:1,000, rabbit; Clontech Laboratories, Inc.), anti-IP3R (1:3,000, mouse; BD Biosciences), and anti-IP3R1 (1:2,000, 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 oviductal ciliated cells stimulated with 20% dextran or 30% hypotonic solutions. Fig. S3 shows calcium imaging data obtained from hamster oviductal 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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