IP_3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5′-6′-epoxyeicosatrienoic acid

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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 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).

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Abbreviations used in this paper: 4α-PDD, 4α-phorbol 12,13-didecanoate; AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; ANOVA, analysis of variance; CBF, ciliary beat frequency; EET, epoxyeicosatrienoic acid; IP_3, inositol trisphosphate; IP_3R, IP_3 receptor; pBPB, 4-bromophenacyl bromide; PLA_2, phospholipase A_2; TRP, transient receptor potential; TRPV4, TRP vanilloid 4.

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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_3R).

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 arachidonyl trifluoromethyl ketone ($AACOCF_3$; 50 μM) also blocked dextran-induced TRPV4 currents (20% dextran: -14.8 ± 0.8 pA/pF, $n = 7$; vs. dextran + $AACOCF_3$: -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 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 Fig. 2 (A–C) are shown in Fig. 2 D. GDPβ-s also reduced the hypotonicity-induced currents to the same levels recorded in

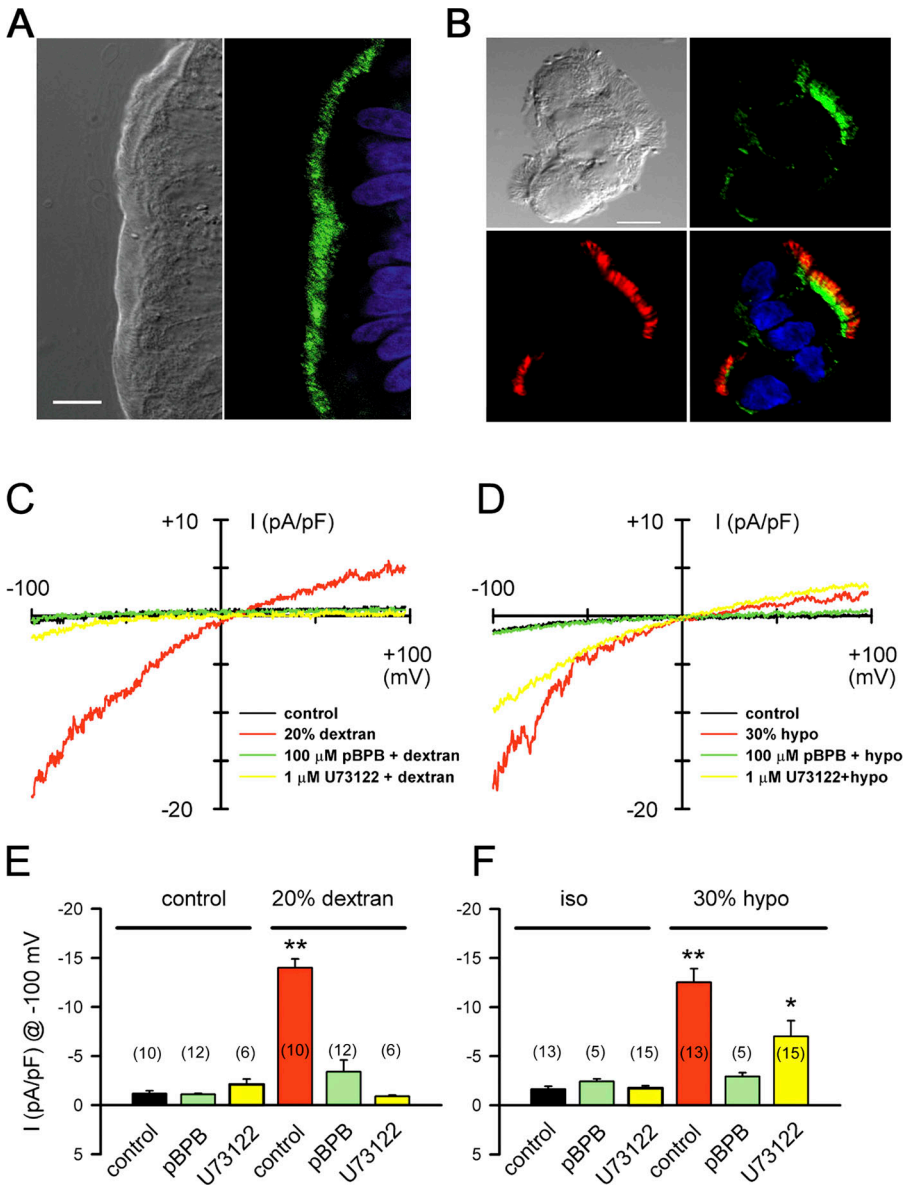

Figure 1. **Localization and activity of TRPV4 channel in hamster oviductal ciliated cells.** (A) Nomarsky image (left) and confocal immunofluorescence image (right) of TRPV4 (green) and TO-PRO-3 nuclear staining (blue) in a tissue section of hamster oviduct. (B) Nomarsky (top left), TRPV4 (top right), α-tubulin (bottom left, red), and merge (bottom right) images obtained from a small cluster of five isolated cells. Colocalization of TRPV4 and tubulin is shown in yellow. Bars, 10 μm. (C) Whole-cell current responses obtained from ciliated cells dialyzed with NMDG-Cl solutions and bathed in control (black), 20% dextran (red), 20% dextran + 100 μM pBPB (green), and 20% dextran + 1 μM U73122 (yellow) solutions. (D) Whole-cell currents were recorded in ciliated cells exposed to isotonic (black), 30% hypotonic (red), hypotonic + 100 μM pBPB (green), and hypotonic + 1 μM U73122 (yellow) solutions. Control and dextran- or hypotonicity-induced currents were recorded from the same cells, whereas currents in the presence of inhibitors were recorded from different cells. (E and F) Mean current density measured at −100 mV. Data are expressed as the mean ± SEM. **, $P < 0.001$; *, $P < 0.05$ compared with control, untreated cells (one-way ANOVA and Bonferroni post hoc).

the presence of U73122 (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200712058/DC1) but significantly different from those obtained in the presence of GDPβ-s and dextran solutions ($P < 0.05$; Fig. 2 D), confirming the differences in sensitivity of high viscosity– and hypotonicity-induced TRPV4 response to the G protein–PLC 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.

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 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). Note that in the

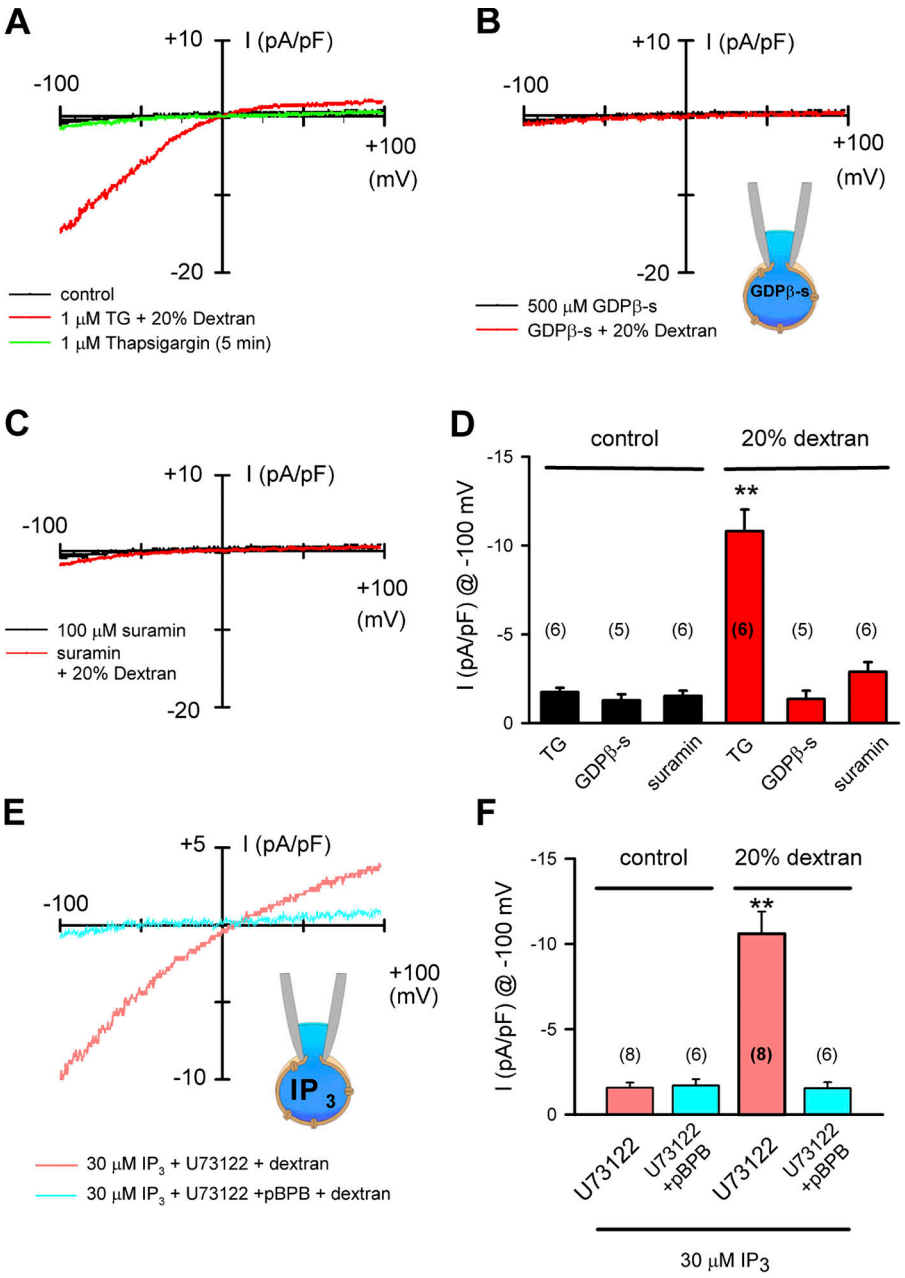

Figure 2. **IP_3-mediated modulation of TRPV4 response to high viscous solutions in hamster oviductal ciliated cells.** (A) Whole-cell currents recorded before (control) and after a 5-min treatment with 1 μM thapsigargin and thapsigargin + 20% dextran. (B) Currents recorded with a pipette solution containing 500 μM GDPβ-s before and after exposure to 20% dextran solutions. (C) Current response to 20% dextran in the presence of 100 μM suramin. (D) Mean current density measured at −100 mV. (E) Currents recorded from cells dialyzed with 30 μM IP_3 and bathed either with U73122 + 20% dextran solution (pink) or U73122+pBPB+dextran (blue). (F) Mean current density measured at −100 mV. Data are expressed as the mean ± SEM. **, $P < 0.001$ (one-way ANOVA and Bonferroni post hoc).

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.

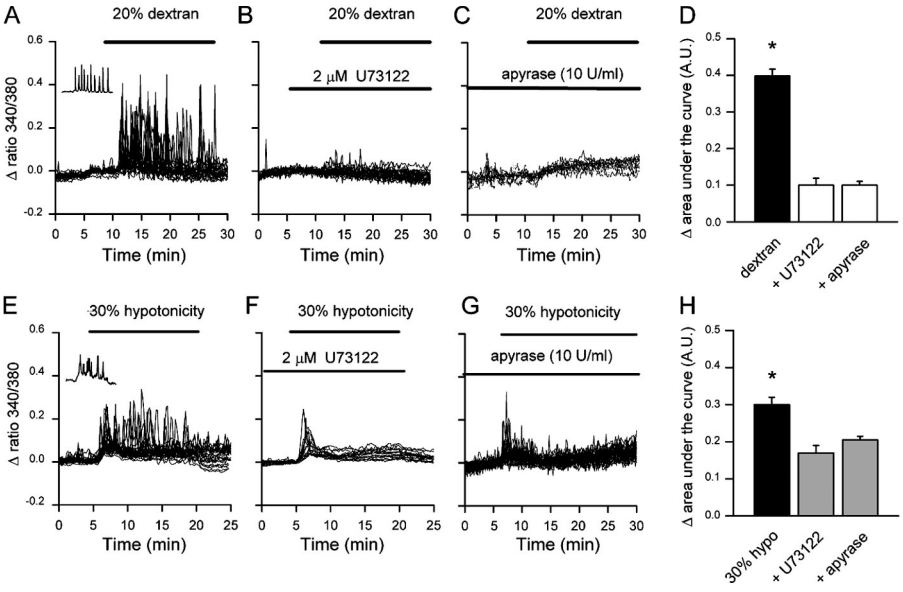

Figure 3. **Effect of PLA_2 and PLC inhibitors on intracellular Ca^{2+} signals.** Representative intracellular Ca^{2+} signals (Δ ratio, 340/380) obtained from different primary cultures of hamster oviductal ciliated cells stimulated with: (A) 20% dextran solution (n = 21; inset shows a recording obtained from a single cell); (B) dextran + 2 μM U73122 (n = 22); and (C) dextran + apyrase (10 U/ml; n = 19). (D) Mean [Ca^{2+}] increases (Δ area under the curve) in response to 20% dextran. Intracellular Ca^{2+} 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 [Ca^{2+}] 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).

IP_3 sensitizes TRPV4 response to physical stimuli that did not reach the threshold level of PLA_2–EET pathway activation

Based on the different effects of PLA_2 and PLC pathway inhibitors on TRPV4-like current and Ca^{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_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 PLA_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_3 pathway. In addition, IP_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 PLA_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 PLA_2 activity in hamster oviductal ciliated cells, we measured the Ca^{2+} signal and its dependence on PLC–IP_3 in ciliated cells under milder hypotonic stimuli (15%), aiming to elicit less PLA_2 activation. Fig. 5 (A and B) shows that, unlike the Ca^{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_3 pathway also becomes crucial to the generation of the Ca^{2+} signal under conditions of lower PLA_2 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 EC_{50} of 3.2 ± 1.2 nM. TRPV4 channel response to 1 nM 5′,6′-EET was greatly potentiated by the presence of 30 μM IP_3, a concentration that elicits maximal activation of IP_3R type 1 (IP_3R1) and 3 (IP_3R3; 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_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′,6′-EET (Fig. 5 D).

IP_3R mediates IP_3-dependent sensitization of TRPV4 to 5′,6′-EET

Next, we further investigated the mechanism of IP_3-mediated sensitization. A previous study has localized IP_3R3 (not IP_3R1) to the plasma membrane of hamster oviduct ciliated cells and suggested its participation in Ca^{2+} influx (Barrera et al., 2004). To test whether IP_3-mediated sensitization was a general mechanism rather than circumscribed to ciliated cells, we expressed

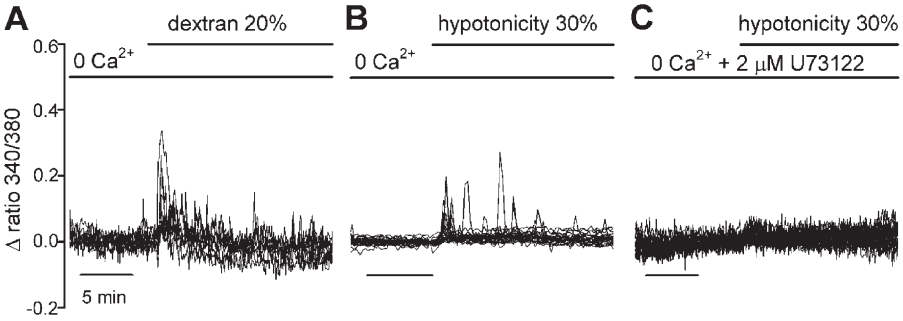

Figure 4. **Effect of removing extracellular Ca^{2+} on intracellular Ca^{2+} signals.** (A) Representative intracellular Ca^{2+} signals obtained from primary cultures of hamster oviductal ciliated cells bathed in Ca^{2+}-free solutions and stimulated with 20% dextran (n = 32), 30% hypotonicity (B; n = 41) and 30% hypotonicity in the presence of 2 μM U73122 (C; n = 25).

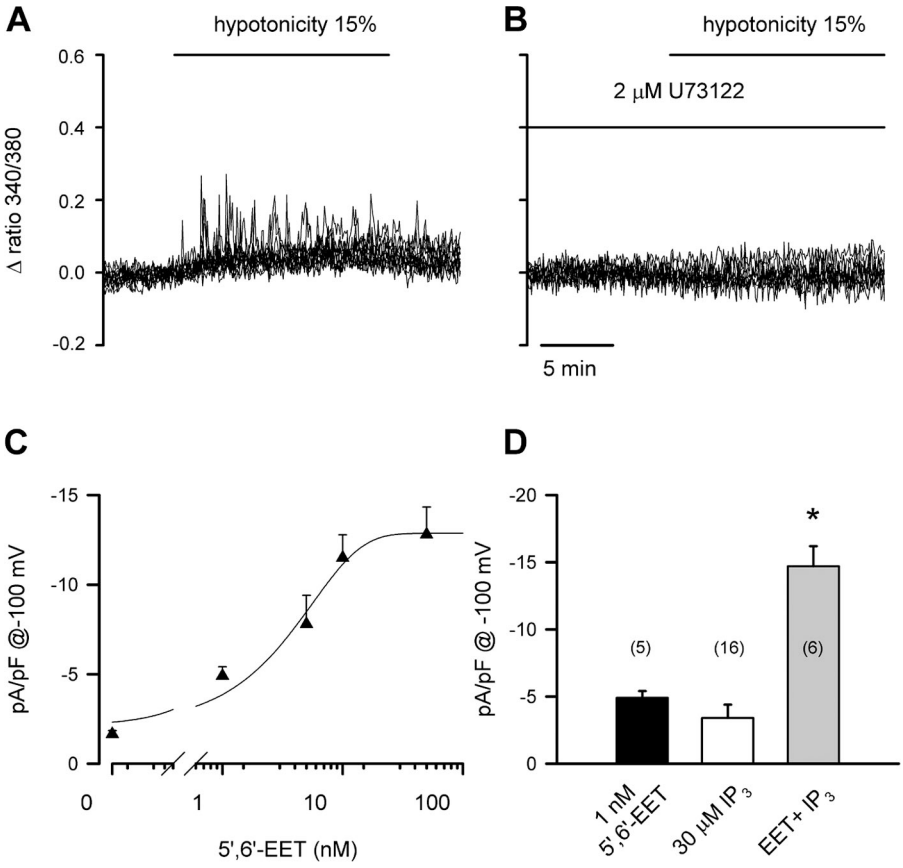

Figure 5. **5′,6′-EET sensitivity of TRPV4 channel in hamster oviduct ciliated cells.** Intracellular Ca^{2+} signals elicited by 15% hypotonic solutions in the absence (A) and presence (B) of the PLC inhibitor U73122 (n = 13 for both conditions). (C) Dose response of TRPV4 currents recorded in ciliated cells loaded with different concentrations of 5′,6′-EET ($n \geq 6$ for each concentration). (D) Potentiation of the TRPV4 response in ciliated cells loaded with 1 nM 5′,6′-EET and 30 μM IP_3. Data are expressed as the mean ± SEM; *, $P < 0.001$ for EET + IP_3 versus EET or IP_3 alone (one way ANOVA and Bonferroni post hoc).

human TRPV4 in HeLa cells that endogenously expressed IP_3R1 and IP_3R3 (Tovey et al., 2001).

Dialysis, through the patch pipette, of HeLa cells expressing human TRPV4 with 1 nM 5′,6′-EET and 30 μM IP_3 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 IP_3 alone elicited no significant TRPV4-like currents (Fig. 6 D). The cationic currents shown in Fig. 6 (A and D) illustrate that the IP_3-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 IP_3 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 IP_3 (Fig. 6 H) or 100 nM 5′,6′-EET alone (Fig. S4). No sensitization was observed in HeLa cells transfected with rat IP_3R3 (Fig. 6, C and F) or EGFP alone (Fig. 6 G). Coexpression of TRPV4 and IP_3R3 (Fig. 6, B, E, and I) tripled IP_3 potentiation as compared with TRPV4 expression alone, an effect that was completely inhibited in the presence of 1 μM of the IP_3R inhibitor xestospongin C. Neither EET (1 nM) nor IP_3 alone activated TRPV4 currents in HeLa cells expressing any combination of EGFP, TRPV4, or IP_3R3 ($P > 0.05$).

As an independent test for the role of PLC–IP_3 signaling in the sensitization of TRPV4, we evaluated the TRPV4-mediated Ca^{2+} 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 IP_3R3. 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 Ca^{2+} signal. In the absence of extracellular ATP, the second hypotonic shock generated a smaller Ca^{2+} signal (Fig. 7 D). Neither hypotonicity-induced Ca^{2+} signal or its enhancement by ATP were observed in HeLa cells overexpressing IP_3R3 alone (Fig. 7 F). The addition of 1 μM ATP alone was insufficient to elicit a Ca^{2+} signal, although cells responded to 100 μM ATP (Fig. 7 C). The presence of low concentrations of extracellular ATP was also essential to record Ca^{2+} signals in response to 20% dextran solutions in HeLa cells expressing TRPV4 and IP_3R3 (Fig. 7 B). In the absence of ATP, no Ca^{2+} 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 IP_3R3 showed no response to dextran solutions containing 1 μM ATP (Fig. 7 G). Mean increases in the Ca^{2+} 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–IP_3

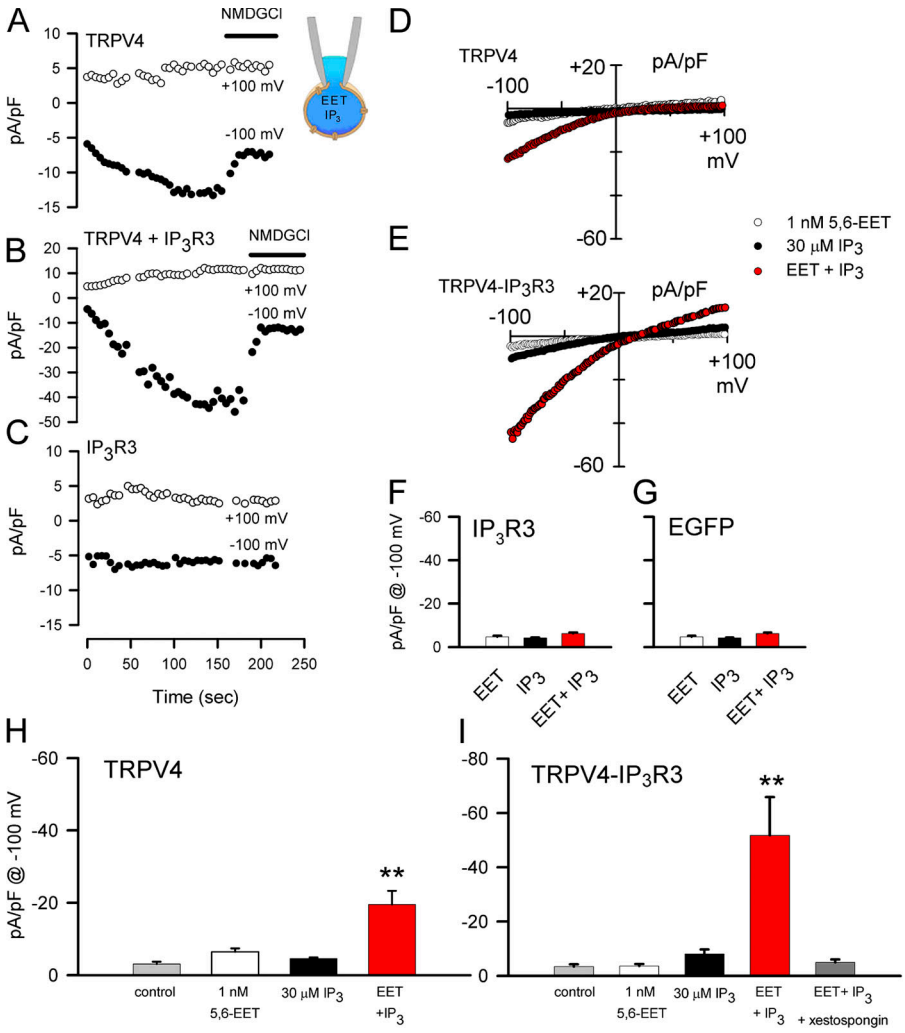

Figure 6. **IP_3R involvement in IP_3-dependent sensitization of TRPV4 to 5′,6′-EET.** Time course of whole-cell cationic currents at –100 (●) and 100 mV (○) in HeLa cells transfected with human TRPV4 (A), TRPV4 and rat IP_3R3 (B), or IP_3R3 (C) and dialyzed with 1 nM 5′,6′-EET and 30 μM IP_3. At the 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 IP_3R3 (E) and dialyzed with either 1 nM 5′,6′-EET, 30 μM IP_3, or EET+IP_3. (F) Current densities at −100 mV in HeLa cells transfected with IP_3R3 and dialyzed with 1 nM 5′,6′-EET (n = 4), 30 μM IP_3 (n = 5), and EET+IP_3 (n = 4). (G) EGFP transfected cells dilayzed with 1 nM 5′,6′-EET (n = 3), 30 μM IP_3 (n = 4), and EET+IP_3 (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 IP_3 (n = 8); and EET + IP_3 (n = 8). (I) Current densities at −100 mV in HeLa cells expressing TRPV4 and IP_3R3: control (n = 6); 1 nM 5′,6′-EET (n = 10); 30 μM IP_3 (n = 11); EET + IP_3 (n = 8); and EET + IP_3 + 1 μM xestospongin C (n = 4). Data are expressed as the mean ± SEM; **, P < 0.001 (one-way ANOVA and Bonferroni post hoc).

signaling sensitizes TRPV4 response to high-viscosity and hypotonic solutions (calcium imaging data) or to the mechano- and osmotransducing TRPV4-activating messenger 5′,6′-EET (patch-clamp data), an effect that depends on the presence of a functional IP_3R.

To examine whether the modulatory effect of IP_3R3 involved a direct association between the receptor and the channel, we immunoprecipitated TRPV4 (using an anti-GFP antibody that recognize YFP) from Hela cells transfected TRPV4-YFP and IP_3R3 but not from nontransfected cells (Fig. 8). Expression of the proteins of interest was probed by Western blotting the same cell lysates (offered) with anti-GFP, anti-IP_3R3, and anti-IP_3R1 antibodies (Fig. 8, top left). TRPV4 coimmunoprecipitated IP_3R3 but not IP_3R1 (Fig. 8, bottom left). Similarly, IP_3R3 also coimmunoprecipitated TRPV4 (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200712058/DC1). No coimmunoprecipitation of IP_3R3 was detected in HeLa cells transfected with YFP alone (Fig. 8, right).

IP_3 does not sensitize TRPV4 to warm temperature

We also evaluated whether IP_3 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-IP_3R3–expressing HeLa cells (Fig. 9 A). However, unlike the EET-induced TRPV4 activation, warm temperature response was unaffected by IP_3 (Fig. 9 B). Current-voltage traces and mean responses to warm temperatures in the presence or absence of 30 μM IP_3 in the pipette solution are shown in Fig. 9 (C and D).

Discussion

In ciliated epithelia, Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} influx pathway and its implication in the generation of the Ca^{2+} signal is essential to comprehend ciliated epithelia's response to mechanical stimulation in the context of both physiological

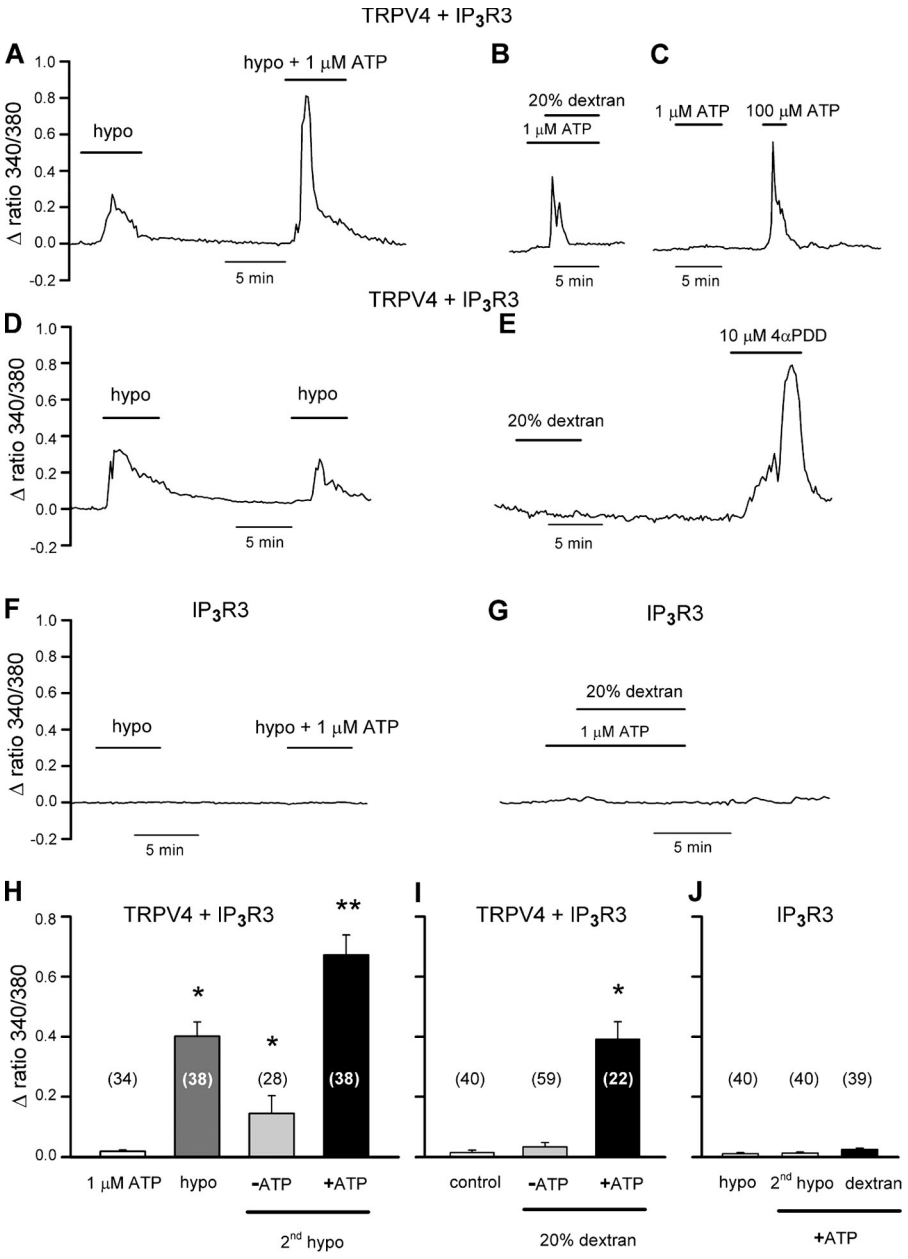

Figure 7. **TRPV4 response to high viscous and hypotonic solutions is potentiated by ATP.** Representative intracellular Ca^{2+} signals (Δ ratio, 340/380) obtained from cells transfected with the indicated constructs and exposed to the conditions shown in the bars. (H–J) Mean increases in 340/380 signal 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) and or double asterisk (hypo + ATP *vs* hypo; one way ANOVA and Bonferroni post hoc).

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 hypotonicicty-induced cationic currents in ciliated cells coincides with the features of cationic currents induced by the TRPV4-specific agonist 4α-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-viscocity 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

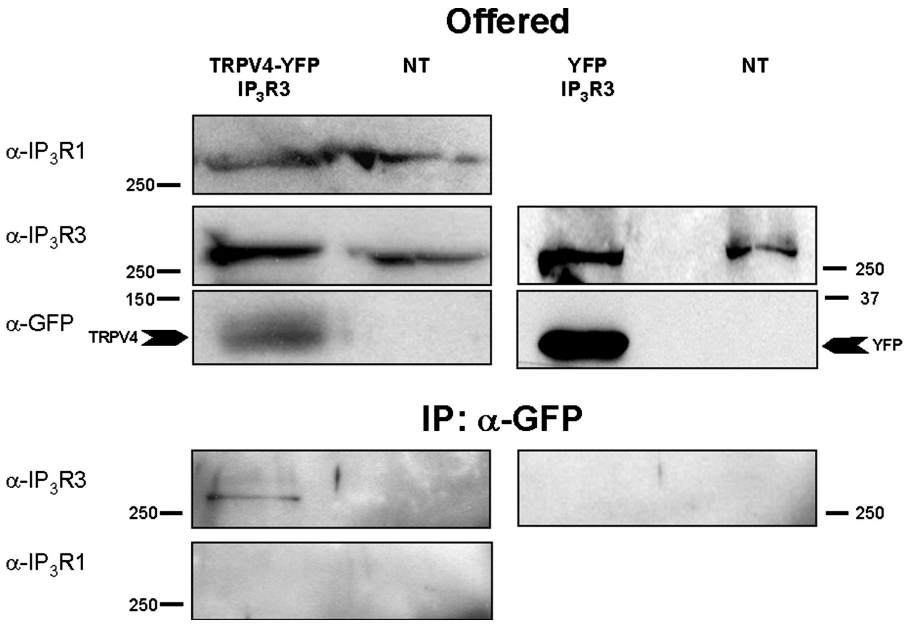

Figure 8. **TRPV4 coimmunoprecipitates with IP_3R3.** (top left) Expression of IP_3R1, IP_3R3, and TRPV4-YFP (arrow) in TRPV4-YFP + IP_3R3 transfected and nontransfected HeLa cells (offered). (bottom left) TRPV4 was immunoprecipitated with anti-GFP polyclonal antibody and immunocomplexes were analyzed by Western blots with either anti-IP_3R3 or anti-IP_3R1 (30-s exposure). (right) Expression of IP_3R3 (top) and YFP (middle) in nontransfected and YFP + IP_3R3 transfected HeLa cells (offered). (bottom) YFP was immunoprecipitated with anti-GFP polyclonal antibody and immunocomplexes were analyzed by Western blots with anti-IP_3R3. Numbers to the sides of the gel blots indicate molecular mass in kD.

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–IP_3 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 $P2Y_2$ 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 IP_3 alone is able to compensate the inhibitory effect of U73122; (3) that the effect of IP_3 requires a functional IP_3R (as the sensitizing effect is

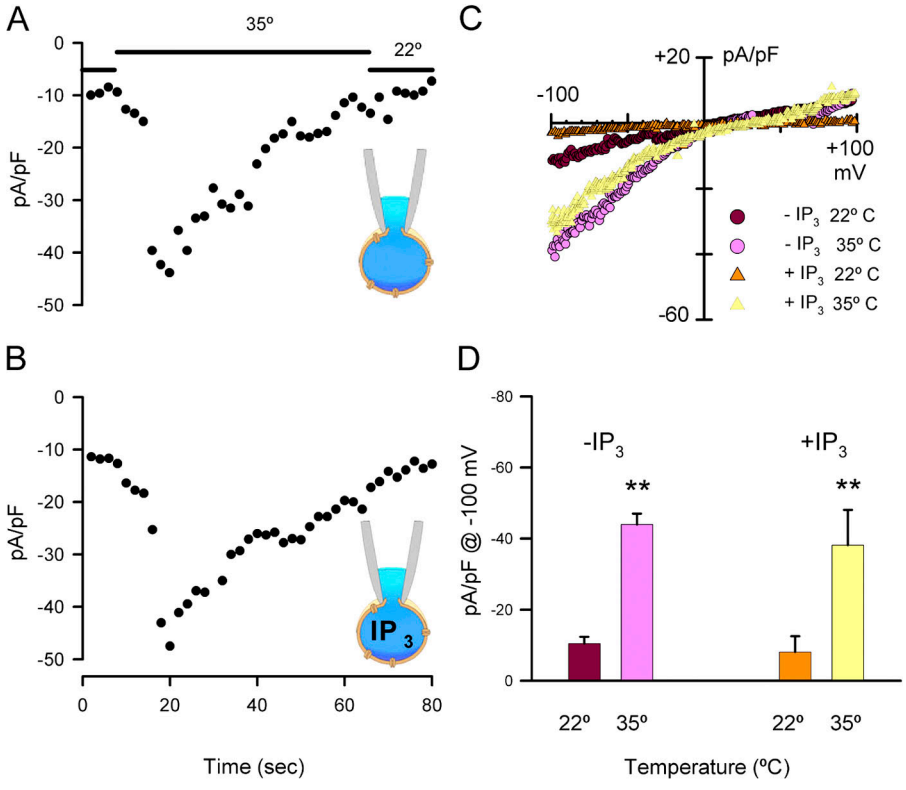

Figure 9. **Effect of IP_3 on TRPV4 response to mild temperature.** Time course of whole-cell cationic currents at –100 mV in HeLa cells transfected with human TRPV4 and rat IP_3R3 exposed to 22 and 35°C in the absence (A) or presence (B) of 30 μM IP_3. (C) Current-voltage relations of whole-cell cationic currents in HeLa cells expressing human TRPV4 and rat IP_3R3 exposed to 22 and 35°C in the absence or presence of 30 μM IP_3 in the pipette solution. (D) Temperature-dependent increase in TRPV4 current density (measured at the peak response) in HeLa cells transfected with TRPV4 and IP_3R3 and recorded in the absence or presence of 30 μM IP_3 in the pipette solution (n = 6 for each condition). Data are expressed as the mean ± SEM; **, $P < 0.001$ (one-way ANOVA and Bonferroni post hoc).

inhibited by xestospongin C) although it does not require the release of Ca^{2+} via IP_3R, 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 4α-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 signaling 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+} dependency of the IP_3R (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_3R 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_3R. 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_3R demonstrated by the coimmunoprecipitation studies is required to support the potentiating effect of IP_3. IP_3Rs 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; Kiselyov et al., 2005; Li et al., 2005), although no description of such mechanisms exists for the subfamily of TRPV channels (Clapham, 2003). Thus, IP_3R, 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_3R 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), 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

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., 2005). 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., 2006): 0.3 µg pEGFPN1, 1.5–3 µg pcDNA-3-TRPV4-human, and 1.5–3 µg rat pcDNA-3-rat IP_3R3s (provided by H. DeSmedt, Katholieke Universiteit Leuven, Leuven, Belgium; and G.I. Bell, University of Chicago, Chicago, IL).

Laser confocal immunodetection

Hamster oviducts were fixed overnight at 4°C with 4% paraformaldehyde + 3.7% sucrose, embedded into 7.5% gelatin, and sectioned with a criostat (~8 µm). Isolated cells were fixed in suspension with 4% (wt/vol) paraformaldehyde + 3.7% sucrose in PBS for 30 min at 4°C and attached to 1.5% gelatin-coated coverslips by spinning at 500 rpm for 3 min using a cytospin (Shandon; Thermo Fisher Scientific). After the spinning, fixation procedure continued for an additional 10 min at RT. Tissue sections and single cells were permeabilized with Tween 20 (0.1% or 0.05%, respectively) in PBS (1 h or 15 min at RT) and nonspecific interactions were blocked with 1.5% BSA + 5% FBS + Tween 20 (0.1% or 0.05%) in PBS. Sections and isolated cells were incubated overnight at 4°C with the primary antibodies diluted in the same blocking solution. For competition experiments, the antigen was incubated with TRPV4 antibody (5:1) for 1 h at RT.

The anti-TRPV4 polyclonal antibody (Arniges et al., 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 (488 nm; JDS Uniphase Corporation) and HeNe (555 and 633 nm; JDS Uniphase Corporation and LASOS Lasertechnik GmbH, respectively) lasers. Original images were not further processed except for adjustments of brightness, contrast, and color balance.

Measurement of intracellular Ca^{2+}

Cytosolic Ca^{2+} 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 Ca^{2+} 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 $MgCl_2$, 5 mM EGTA, 10 mM Hepes, 4 mM ATP, and 0.1 mM GTP (300 mosmoles/liter, pH 7.3). Occasionally, pipette solutions contained different concentrations of 5′,6′-EET and IP_3 (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 IP_3R3 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 by resuspension in BNP40 lysis buffer (1% Nonidet P-40, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 0.05% aprotinin, pH 7.4, and 50 mM Tris-Cl, pH 7.4) containing 12% of additional protease inhibitor cocktail (Roche). Cell extracts were agitated for 1 h at 4°C, aspirated three times through a 25-gauge needle, and centrifuged at 13,000 rpm for 1 h at 4°C. Protein concentration was quantified by the Bradford method. 620 µg of total protein samples from transfected and nontransfected HeLa cells were 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-IP_3R3 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-IP_3R3 (1:3,000, mouse; BD Biosciences), and anti-IP_3R1 (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 GDPβ-s, U73122, IP_3, 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 IP_3 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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