

IP₃ sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid

Jacqueline Fernandes, Ivan M. Lorenzo, Yaniré N. Andrade, Anna Garcia-Elias, Selma A. Serra, José M. Fernández-Fernández, and Miguel A. Valverde

Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona 08003, Spain

Mechanical and osmotic sensitivity of the transient receptor potential vanilloid 4 (TRPV4) channel depends on phospholipase A₂ (PLA₂) 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 PLA₂-EET as the primary pathway to activate TRPV4. Under conditions of low PLA₂ activation, both also use extracellular ATP-mediated activation of phospholipase C (PLC)-inositol trisphosphate (IP₃) signaling to support TRPV4 gating. IP₃, without being

an agonist itself, sensitizes TRPV4 to EET in epithelial ciliated cells and cells heterologously expressing TRPV4, an effect inhibited by the IP₃ receptor antagonist xestospingin C. Coimmunoprecipitation assays indicated a physical interaction between TRPV4 and IP₃ receptor 3. Collectively, our study suggests a functional coupling between plasma membrane TRPV4 channels and intracellular store Ca²⁺ channels required to initiate and maintain the oscillatory Ca²⁺ signal triggered by high viscosity and hypotonic stimuli that do not reach a threshold level of PLA₂ 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 Sleight, 1990). Both mechanical and chemical stimulation of ciliated cells are linked to the modulation of CBF by an intracellular Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ influx (Sanderson and Dirksen, 1986; Satir and Sleight, 1990; Boitano et al., 1994), release of ATP (Okada et al., 2006; Winters et al., 2007), and inositol trisphosphate (IP₃)-mediated intracellular Ca²⁺ 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 Ca²⁺ signal generated by transient receptor potential vanilloid 4 (TRPV4) channel activation after exposure to high viscous solutions (Andrade et al., 2005).

J. Fernandes and I.M. Lorenzo contributed equally to this paper.

Correspondence to M. Valverde: miguel.valverde@upf.edu

Abbreviations used in this paper: 4 α -PDD, 4 α -phorbol 12,13-didecanoate; AA, arachidonic acid; AACOCF₃, arachidonyl trifluoromethyl ketone; ANOVA, analysis of variance; CBF, ciliary beat frequency; EET, epoxyeicosatrienoic acid; IP₃, inositol trisphosphate; IP₃R, IP₃ receptor; pBPB, 4-bromophenacyl bromide; PLA₂, phospholipase A₂; TRP, transient receptor potential; TRPV4, TRP vanilloid 4.

The online version of this paper contains supplemental material.

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, epoxy-eicosatrienoic 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 (Vandenberg 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 (Vandenberg 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 μ M 4-bromophenacyl bromide (pBPPB) or PLC with 1 μ M 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 pBPPB (Fig. 1 D). Inhibition of PLA₂ with arachidonyl trifluoromethyl ketone (AACOCF₃; 50 μ M) 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 μ M 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 μ M GDP β -s, which locks G proteins in their inactive state (Fig. 2 B), or treated with 100 μ M 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. GDP β -s also reduced the hypotonicity-induced currents to the same levels recorded in

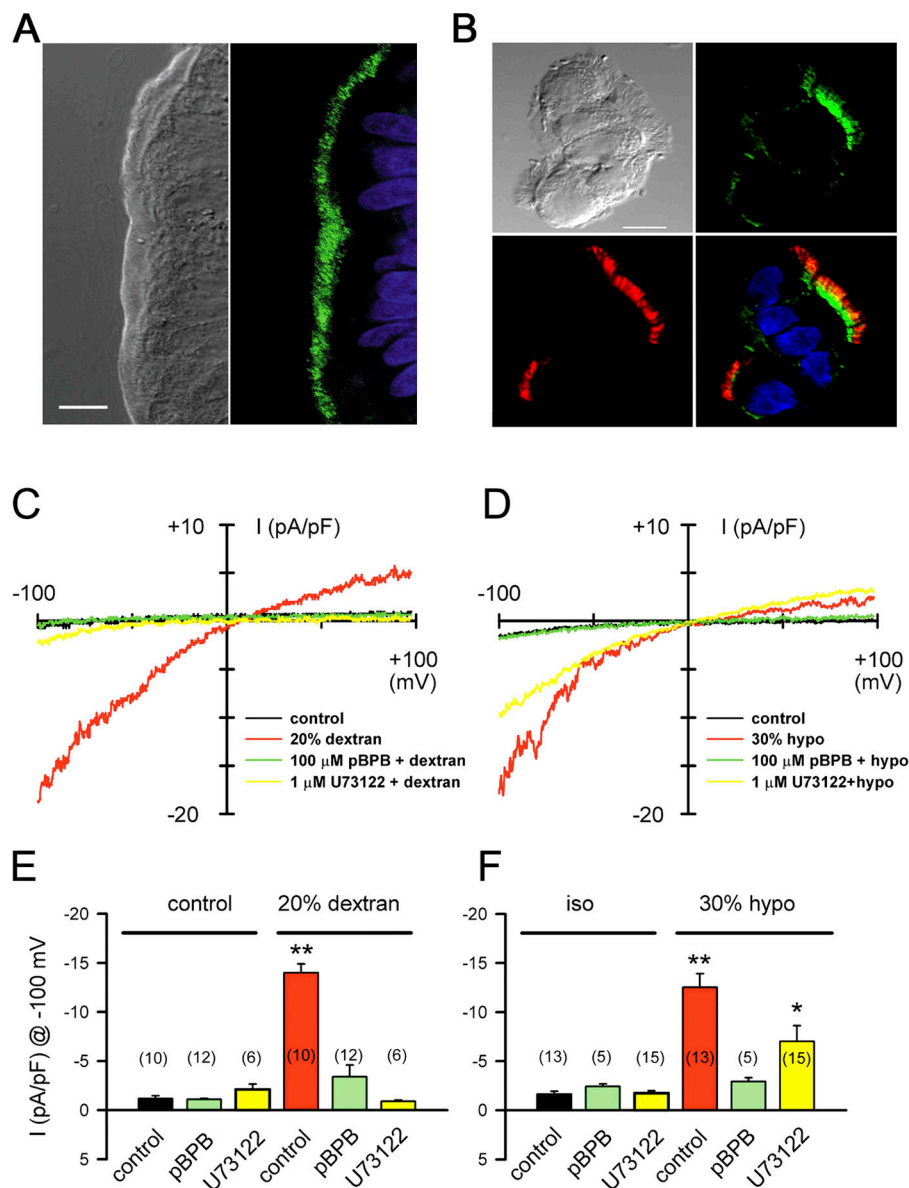
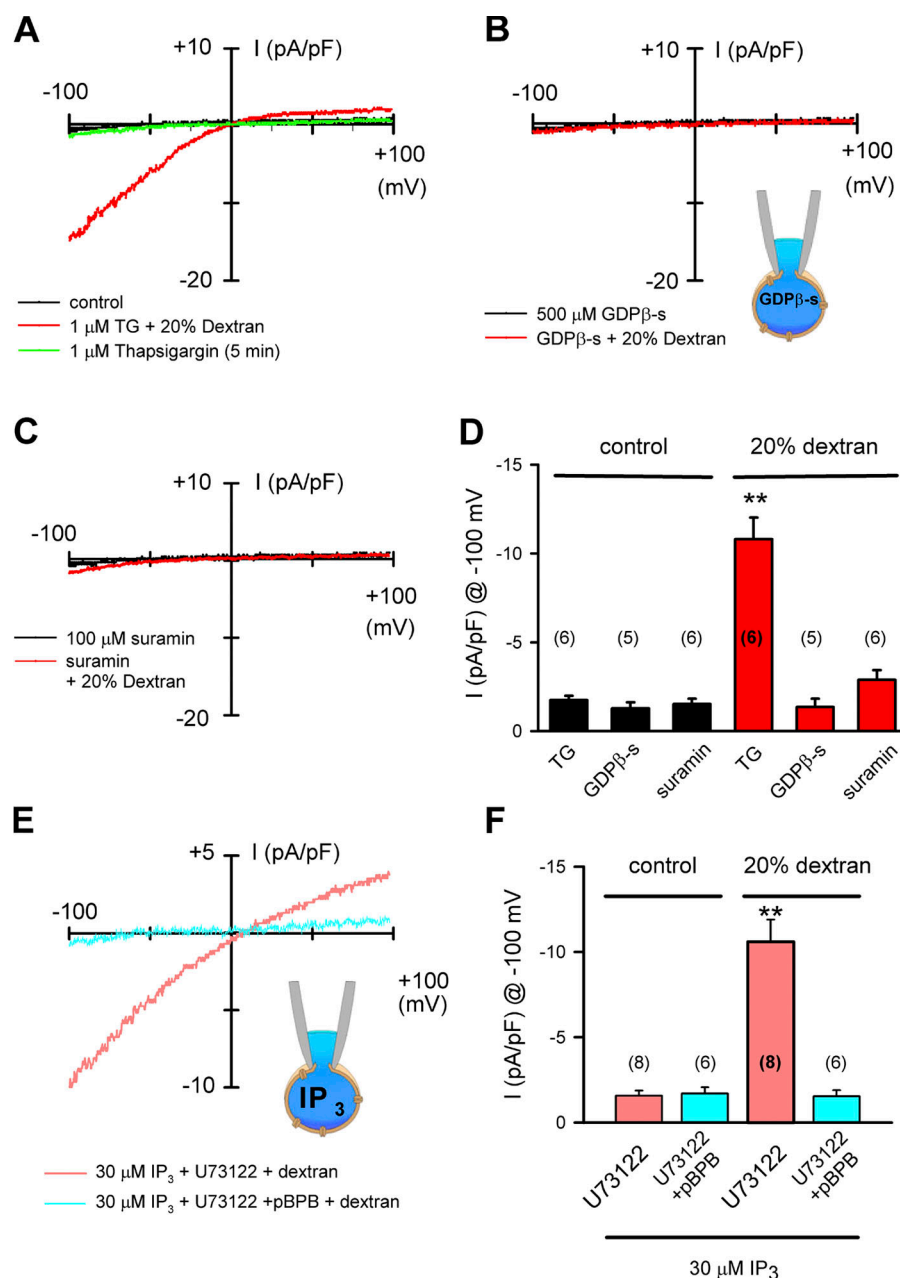


Figure 1. Localization and activity of TRPV4 channel in hamster oviductal ciliated cells. (A) Nomarsky image (left) and confocal immunofluorescence image (right) of TRPV4 (green) and TO-PRO-3 nuclear staining (blue) in a tissue section of hamster oviduct. (B) Nomarsky (top left), TRPV4 (top right), α -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 μ m. (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 μ M pBPB (green), and 20% dextran + 1 μ M U73122 (yellow) solutions. (D) Whole-cell currents were recorded in ciliated cells exposed to isotonic (black), 30% hypotonic (red), hypotonic + 100 μ M pBPB (green), and hypotonic + 1 μ M 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 \pm 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 GDP β -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 μ M IP₃ restored TRPV4 activation by dextran solutions in the presence of U73122 (PLC inhibitor) except when both U73122 and pBPB (PLA₂ inhibitor) were used (Fig. 2, E and F). Similarly, the presence of IP₃ in the pipette solution also restored full TRPV4 activation by 30% hypotonic solutions (Fig. S2 A). The combination of pipette solutions containing IP₃ 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 IP₃-mediated sensitization of TRPV4 response to highly viscous loads.

Consistent with the electrophysiological experiments, inhibition of PLC with U73122 did not modify the basal Ca²⁺ signal but prevented the oscillatory Ca²⁺ signal generated by 20% dextran in primary cultures of hamster oviductal ciliated cells (Fig. 3, A and B; representative single-cell Ca²⁺ signal is shown in Fig. 3 A, inset). U73122 also prevented the oscillatory Ca²⁺ signal generated by 30% hypotonic solutions (Fig. 3, E and F; representative single-cell Ca²⁺ 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-IP₃ signaling (Fig. 3, C and G). A quantitative analysis of the Ca²⁺ signal, calculating the mean area under the curve as an indicator of the magnitude of the Ca²⁺ signal, is shown in Fig. 3 (D and H). Note that in the

Figure 2. IP₃-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 μ M thapsigargin and thapsigargin + 20% dextran. (B) Currents recorded with a pipette solution containing 500 μ M GDP β -s before and after exposure to 20% dextran solutions. (C) Current response to 20% dextran in the presence of 100 μ M suramin. (D) Mean current density measured at -100 mV. (E) Currents recorded from cells dialyzed with 30 μ M IP₃ 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 \pm 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-IP₃, 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 Ca²⁺ signals elicited by high-viscosity and hypotonic solutions. To address this point, we focused on the role of Ca²⁺ entry in the maintenance of the oscillatory signals. Upon withdrawal of extracellular Ca²⁺, 20% dextran (Fig. 4 A) or 30% hypotonic solutions (Fig. 4 B) generated a transient peak followed by oscillating Ca²⁺ 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 Ca²⁺ signal was detected. These responses were very different from those obtained in

the presence of extracellular Ca²⁺ (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 Ca²⁺ peaks observed in Ca²⁺-free solutions (Fig. 4, A and B) originated from intracellular stores, most likely IP₃-sensitive stores, as the signal disappeared in the presence of U73122 (Fig. 4 C). Under conditions where the PLC-IP₃ pathway was intact but ER Ca²⁺ 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 Ca²⁺-containing solutions, the initial Ca²⁺ peaks triggered by 20% dextran and 30% hypotonic solutions appear to involve both Ca²⁺ entry and intracellular release, whereas maintained oscillations after the initial peak were mainly dependent on intracellular Ca²⁺ stores.

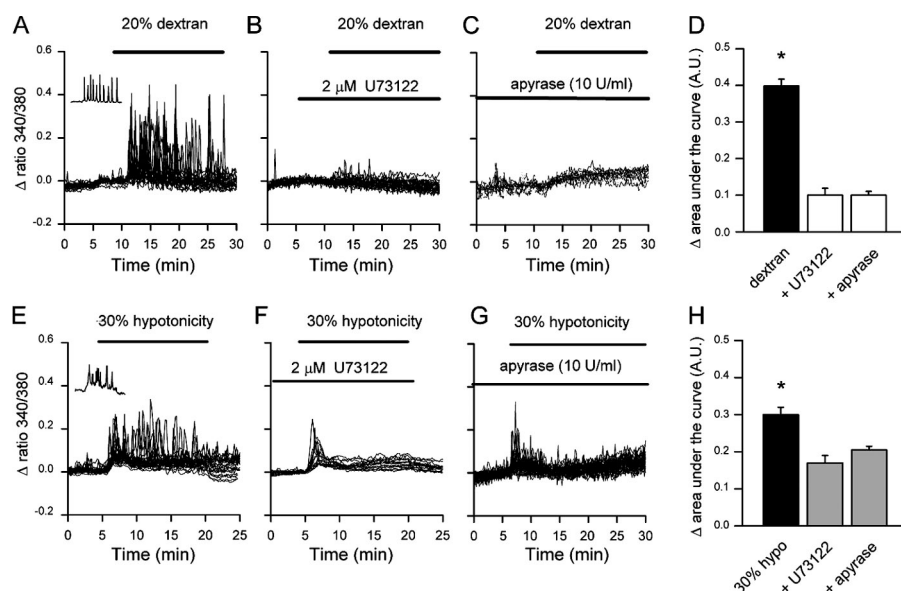


Figure 3. Effect of PLA₂ and PLC inhibitors on intracellular Ca²⁺ signals. Representative intracellular Ca²⁺ signals (Δ ratio, 340/380) obtained from different primary cultures of hamster oviductal ciliated cells stimulated with: (A) 20% dextran solution ($n = 21$; inset shows a recording obtained from a single cell); (B) dextran + 2 μ M 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 μ M 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 \pm SEM; *, $P < 0.05$ for dextran and hypotonicity versus the inhibitors (one way ANOVA and Bonferroni post hoc).

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 posited 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 re-

sponse 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 μ M IP₃, a concentration that elicits maximal activation of 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).

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 IP₃R3 (not IP₃R1) to the plasma membrane of hamster oviduct ciliated cells and suggested its participation in Ca²⁺ influx (Barrera et al., 2004). To test whether IP₃-mediated sensitization was a general mechanism rather than circumscribed to ciliated cells, we expressed

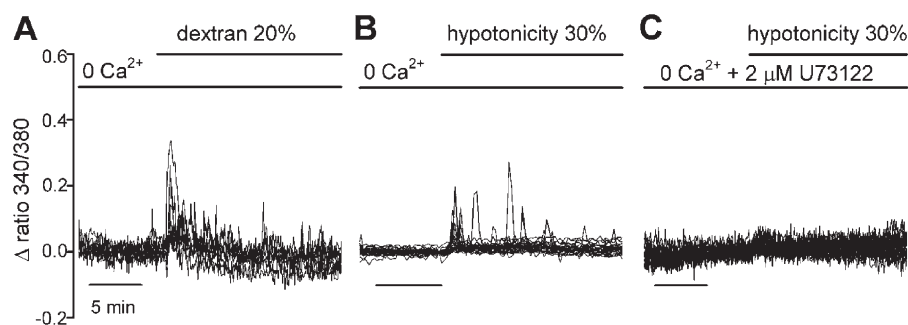
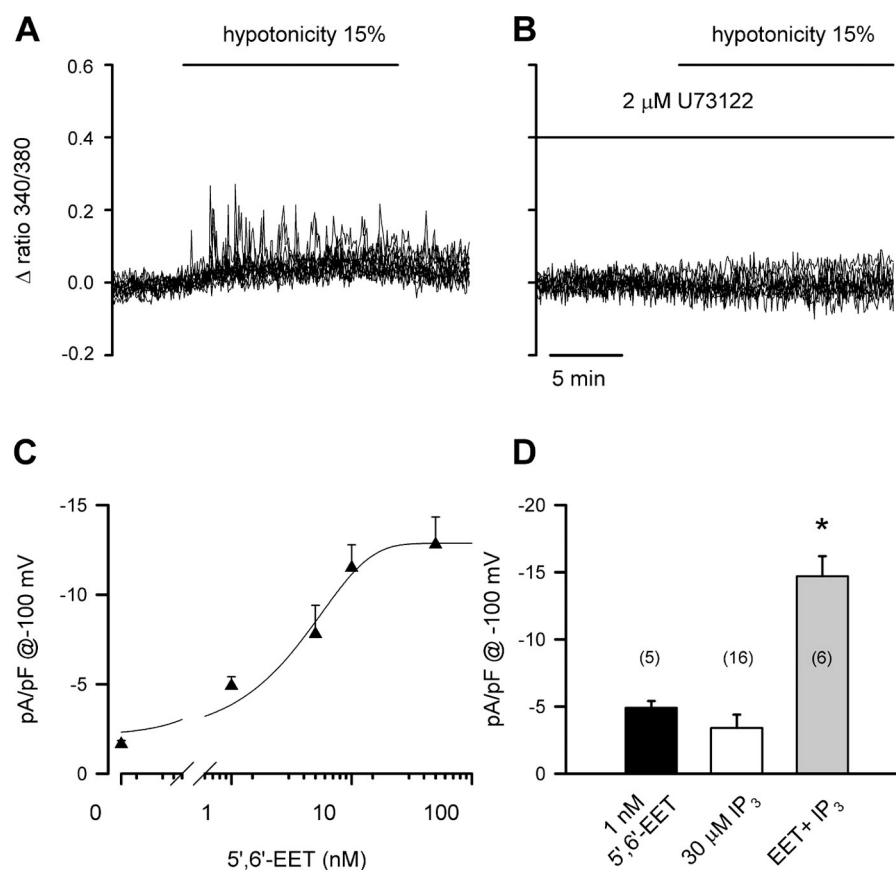


Figure 4. Effect of removing extracellular Ca²⁺ on intracellular Ca²⁺ signals. (A) Representative intracellular Ca²⁺ signals obtained from primary cultures of hamster oviductal ciliated cells bathed in Ca²⁺-free solutions and stimulated with 20% dextran ($n = 32$), 30% hypotonicity (B; $n = 41$) and 30% hypotonicity in the presence of 2 μ M 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 \pm 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 $\text{IP}_3\text{R1}$ and $\text{IP}_3\text{R3}$ (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 $\text{IP}_3\text{R3}$ (Fig. 6, C and F) or EGFP alone (Fig. 6 G). Coexpression of TRPV4 and $\text{IP}_3\text{R3}$ (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 xestospongine C. Neither EET (1 nM) nor IP_3 alone activated TRPV4 currents in HeLa cells expressing any combination of EGFP, TRPV4, or $\text{IP}_3\text{R3}$ ($P > 0.05$).

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 $\text{IP}_3\text{R3}$. 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 $\text{IP}_3\text{R3}$ 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 $\text{IP}_3\text{R3}$ (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 4 α -phorbol 12,13-didecanoate (4 α -PDD; 10 μM). Similar results were obtained in CHO, HEK, and COS cells transfected with human TRPV4 ($n > 200$; unpublished data). HeLa cells expressing $\text{IP}_3\text{R3}$ 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

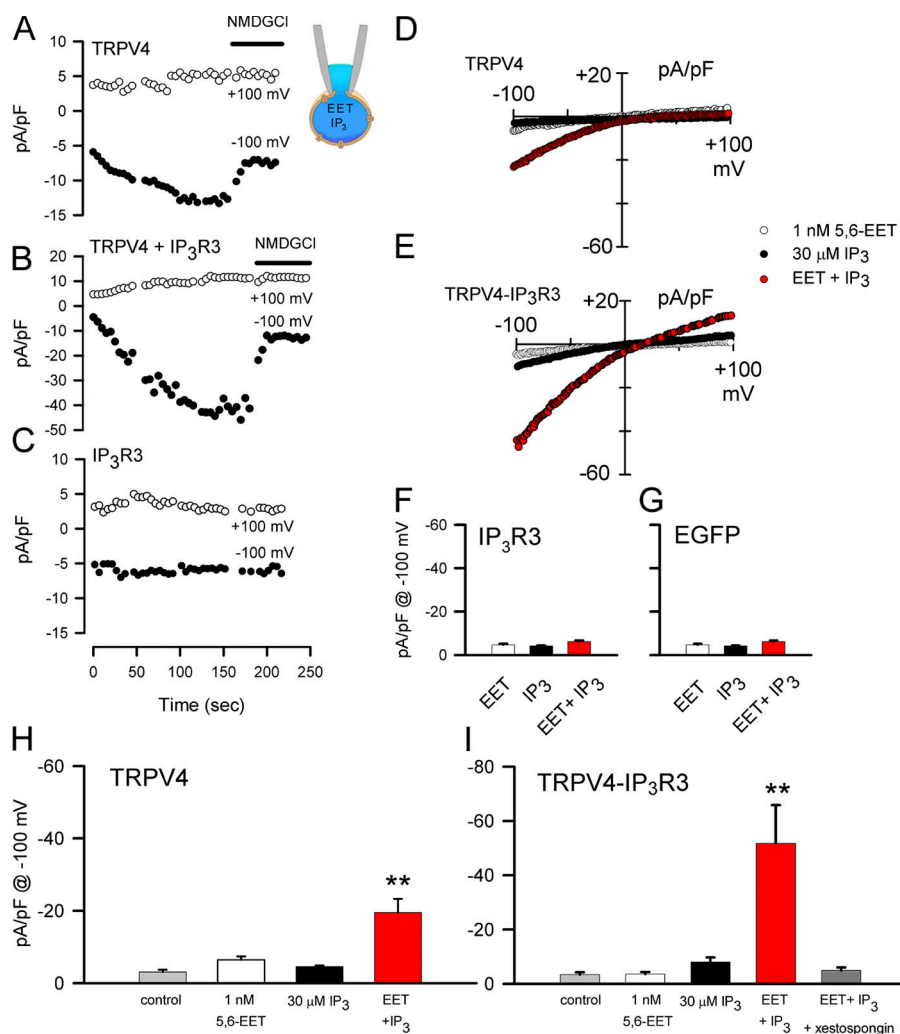


Figure 6. IP₃R involvement in IP₃-dependent sensitization of TRPV4 to 5',6'-EET. Time course of whole-cell cationic currents at -100 mV (●) and 100 mV (○) in HeLa cells transfected with human TRPV4 (A), TRPV4 and rat IP₃R3 (B), or IP₃R3 (C) and dialyzed with 1 nM 5',6'-EET and 30 μ M IP₃. At the time 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 μ M 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 μ M IP₃ ($n = 5$), and EET+IP₃ ($n = 4$). (G) EGFP transfected cells dialyzed with 1 nM 5',6'-EET ($n = 3$), 30 μ M 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 μ M 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 μ M IP₃ ($n = 11$); EET + IP₃ ($n = 8$); and EET + IP₃ + 1 μ M xestospongin C ($n = 4$). Data are expressed as the mean \pm 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 IP₃R.

To examine whether the modulatory effect of IP₃R3 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 IP₃R3 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-IP₃R3, and anti-IP₃R1 antibodies (Fig. 8, top left). TRPV4 coimmunoprecipitated IP₃R3 but not IP₃R1 (Fig. 8, bottom left). Similarly, IP₃R3 also coimmunoprecipitated TRPV4 (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200712058/DC1>). No coimmunoprecipitation of IP₃R3 was detected in HeLa cells transfected with YFP alone (Fig. 8, right).

IP₃ does not sensitize TRPV4 to warm temperature

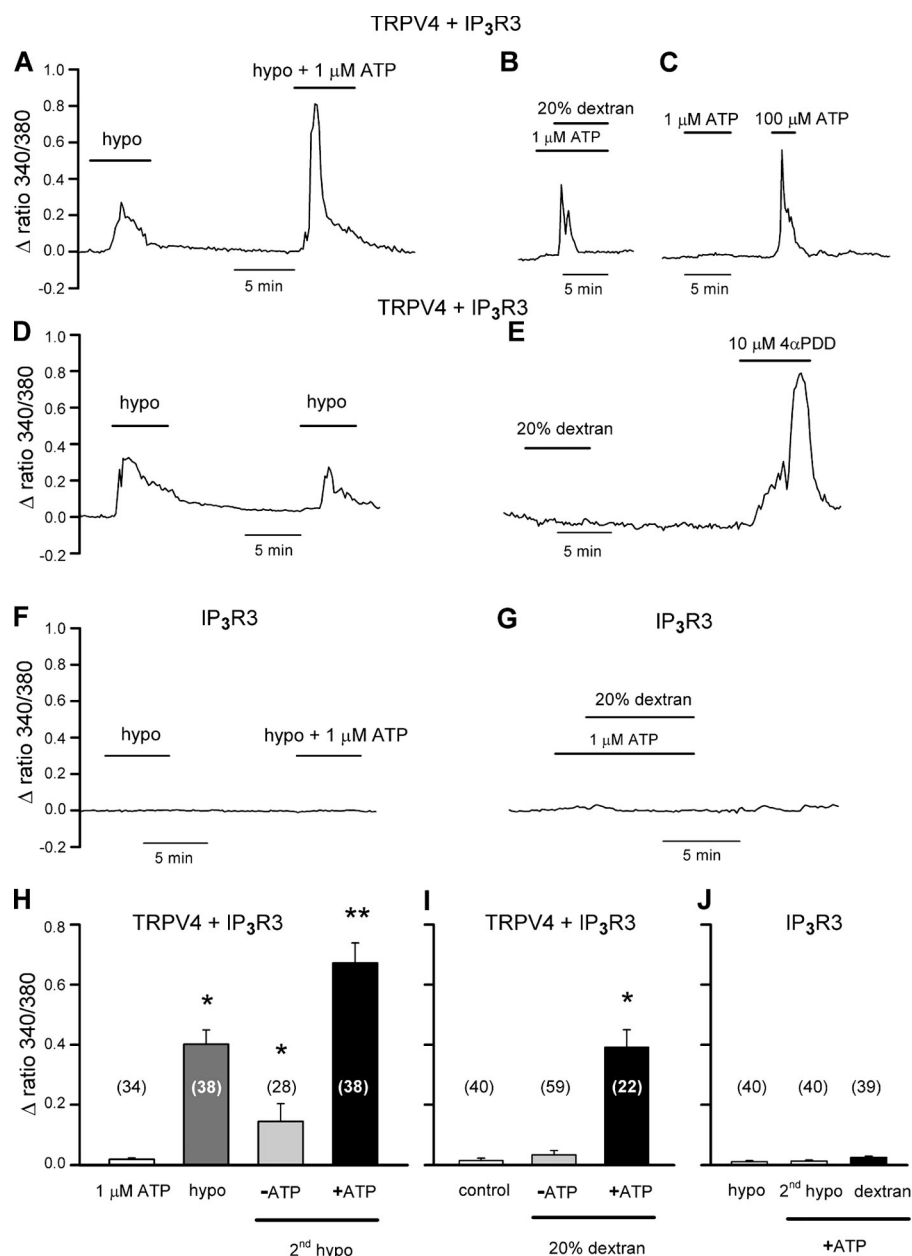
We also evaluated whether IP₃ sensitizes the response of TRPV4 to warm temperature (Guler et al., 2002; Watanabe

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 μ M 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 Sleight, 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

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 \pm SEM of multiple cells recorded from four independent experiments for each condition. Data are expressed as the mean \pm 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 hypotonicity-induced cationic currents in ciliated cells coincides with the features of cationic currents induced by the TRPV4-specific agonist 4 α -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

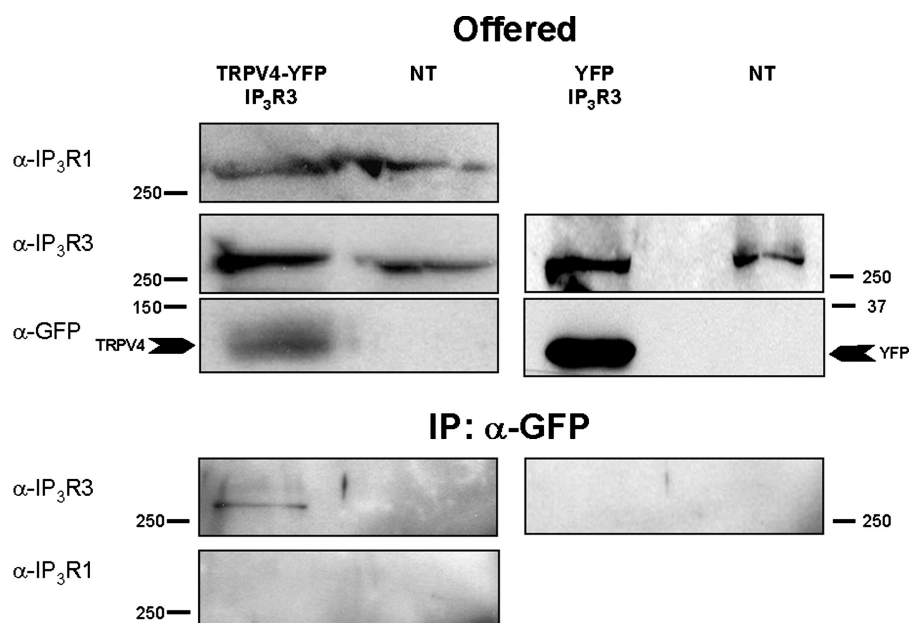


Figure 8. TRPV4 coimmunoprecipitates with IP₃R3. (top left) Expression of IP₃R1, IP₃R3, and TRPV4-YFP (arrow) in TRPV4-YFP + IP₃R3 transfected and nontransfected HeLa cells (offered). (bottom left) TRPV4 was immunoprecipitated with anti-GFP polyclonal antibody and immunocomplexes were analyzed by Western blots with either anti-IP₃R3 or anti-IP₃R1 (30-s exposure). (right) Expression of IP₃R3 (top) and YFP (middle) in nontransfected and YFP + IP₃R3 transfected HeLa cells (offered). (bottom) YFP was immunoprecipitated with anti-GFP polyclonal antibody and immunocomplexes were analyzed by Western blots with anti-IP₃R3. 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-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

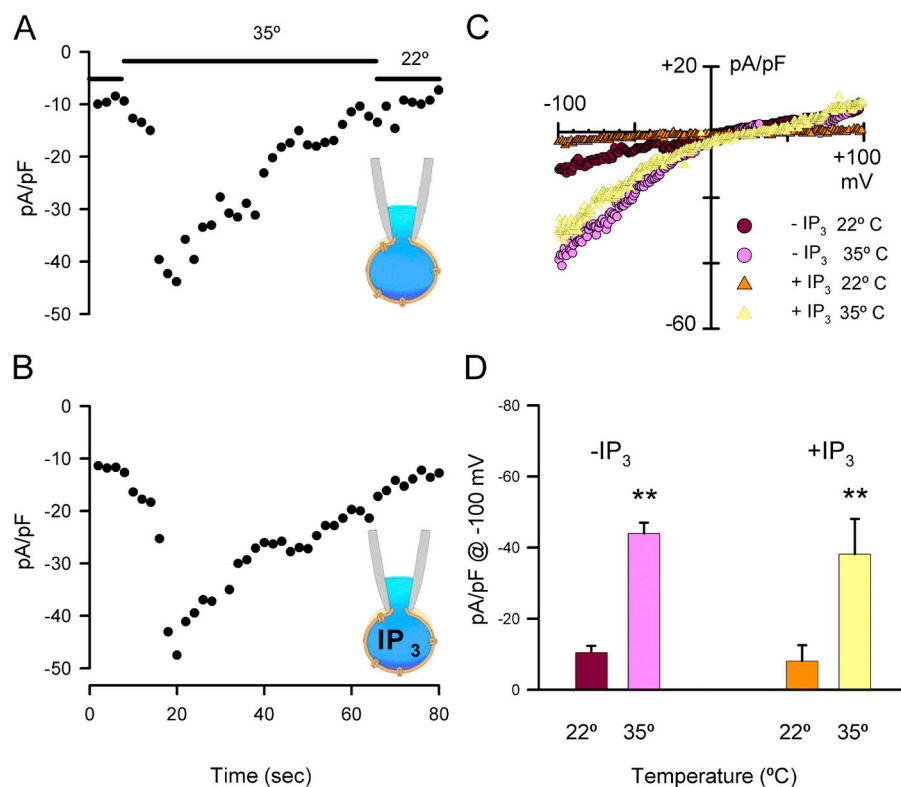


Figure 9. Effect of IP₃ on TRPV4 response to mild temperature. Time course of whole-cell cationic currents at -100 mV in HeLa cells transfected with human TRPV4 and rat IP₃R3 exposed to 22 and 35°C in the absence (A) or presence (B) of 30 μM IP₃. (C) Current-voltage relations of whole-cell cationic currents in HeLa cells expressing human TRPV4 and rat IP₃R3 exposed to 22 and 35°C in the absence or presence of 30 μM IP₃ in the pipette solution. (D) Temperature-dependent increase in TRPV4 current density (measured at the peak response) in HeLa cells transfected with TRPV4 and IP₃R3 and recorded in the absence or presence of 30 μM IP₃ 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 4 α -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), AACOCF₃, and pBBP (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 pEG-FPN1, 1.5–3 µg pcDNA3-TRPV4-human, and 1.5–3 µg rat pcDNA3-rat IP₃R3s (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 cryostat (~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 PI 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 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 mosmoles/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 by resuspension in BNP40 lysis buffer (1% Nonidet P-40, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 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-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-rGFP for TRPV4 detection (1:1,000, rabbit; Clontech Laboratories, Inc.), anti-IP₃R3 (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 GDPβ-s, 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>.

We acknowledge C. Plata, E. Vazquez, and C. Jung for technical support and discussions; and H. DeSmedt and G.I. Bell for rat IP₃R3 cDNA.

This work was supported by grants from the Spanish Ministries of Education and Science (SAF2006-04973 and SAF2006-13893-CO2-02), and Health (Fondo de Investigación Sanitaria, Red HERACLES RD06/0009), the Generalitat de Catalunya (SGR05-266), and Fundació la Marató de TV3 (061331). J.M. Fernández-Fernández is a Ramón y Cajal Fellow. The authors declare that they have no competing financial interests.

Submitted: 11 December 2007

Accepted: 14 March 2008

References

- Afzelius, B.A. 2004. Cilia-related diseases. *J. Pathol.* 204:470–477.
- Alessandri-Haber, N., O.A. Dina, E.K. Joseph, D. Reichling, and J.D. Levine. 2006. A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators. *J. Neurosci.* 26:3864–3874.
- Andrade, Y.N., J. Fernandes, E. Vazquez, J.M. Fernandez-Fernandez, M. Arniges, T.M. Sanchez, M. Villalon, and M.A. Valverde. 2005. TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *J. Cell Biol.* 168:869–874.
- Andrade, Y.N., J. Fernandes, I.M. Lorenzo, M. Arniges, and M.A. Valverde. 2007. The TRPV4 channel in ciliated epithelia. In *TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades*. W. Liedtke and S. Heller, editors. CRC Taylor and Francis, Boca Raton, FL. 413–420.
- Arniges, M., E. Vazquez, J.M. Fernandez-Fernandez, and M.A. Valverde. 2004. Swelling-activated Ca²⁺ entry via TRPV4 channel is defective in cystic fibrosis airway epithelia. *J. Biol. Chem.* 279:54062–54068.
- Arniges, M., J.M. Fernandez-Fernandez, N. Albrecht, M. Schaefer, and M.A. Valverde. 2006. Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking. *J. Biol. Chem.* 281:1580–1586.

- Barrera, N.P., B. Morales, and M. Villalon. 2004. Plasma and intracellular membrane inositol 1,4,5-trisphosphate receptors mediate the Ca^{2+} increase associated with the ATP-induced increase in ciliary beat frequency. *Am. J. Physiol. Cell Physiol.* 287:C1114–C1124.
- Boitano, S., M.J. Sanderson, and E.R. Dirksen. 1994. A role for Ca^{2+} -conducting ion channels in mechanically induced signal transduction of airway epithelial cells. *J. Cell Sci.* 107(Pt 11):3037–3044.
- Boulay, G., D.M. Brown, N. Qin, M. Jiang, A. Dietrich, M.X. Zhu, Z. Chen, M. Birnbaumer, K. Mikoshiba, and L. Birnbaumer. 1999. Modulation of Ca^{2+} entry by polypeptides of the inositol 1,4,5-trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP3R in store depletion-activated Ca^{2+} entry. *Proc. Natl. Acad. Sci. USA.* 96:14955–14960.
- Christensen, A.P., and D.P. Corey. 2007. TRP channels in mechanosensation: direct or indirect activation? *Nat. Rev. Neurosci.* 8:510–521.
- Clapham, D.E. 2003. TRP channels as cellular sensors. *Nature.* 426:517–524.
- De Smet, P., J.B. Parys, S. Vanlinden, G. Bultynck, G. Callewaert, A. Galione, H. De Smedt, and L. Missiaen. 1999. The relative order of IP3 sensitivity of types 1 and 3 IP3 receptors is pH dependent. *Pflugers Arch.* 438:154–158.
- Dellis, O., S.G. Dedos, S.C. Tovey, U.R. Taufiq, S.J. Dubel, and C.W. Taylor. 2006. Ca^{2+} entry through plasma membrane IP3 receptors. *Science.* 313:229–233.
- Evans, J.H., and M.J. Sanderson. 1999. Intracellular calcium oscillations regulate ciliary beat frequency of airway epithelial cells. *Cell Calcium.* 26:103–110.
- Felix, J.A., M.L. Woodruff, and E.R. Dirksen. 1996. Stretch increases inositol 1,4,5-trisphosphate concentration in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 14:296–301.
- Fernandez-Fernandez, J.M., M. Nobles, A. Currid, E. Vazquez, and M.A. Valverde. 2002. Maxi K^+ channel mediates regulatory volume decrease response in a human bronchial epithelial cell line. *Am. J. Physiol. Cell Physiol.* 283:C1705–C1714.
- Gao, X., L. Wu, and R.G. O'Neil. 2003. Temperature-modulated diversity of TRPV4 channel gating: activation by physical stresses and phorbol ester derivatives through protein kinase C-dependent and -independent pathways. *J. Biol. Chem.* 278:27129–27137.
- Grant, A.D., G.S. Cottrell, S. Amadesi, M. Trevisani, P. Nicoletti, S. Materazzi, C. Altier, N. Cenac, G.W. Zamponi, F. Bautista-Cruz, et al. 2007. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. *J. Physiol.* 578:715–733.
- Gudi, S., J.P. Nolan, and J.A. Frangos. 1998. Modulation of GTPase activity of G proteins by fluid shear stress and phospholipid composition. *Proc. Natl. Acad. Sci. USA.* 95:2515–2519.
- Gudi, S., I. Huvar, C.R. White, N.L. McKnight, N. Dusserre, G.R. Boss, and J.A. Frangos. 2003. Rapid activation of Ras by fluid flow is mediated by G α_q and G $\beta\gamma$ subunits of heterotrimeric G proteins in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 23:994–1000.
- Guler, A.D., H. Lee, T. Iida, I. Shimizu, M. Tominaga, and M. Caterina. 2002. Heat-evoked activation of the ion channel, TRPV4. *J. Neurosci.* 22:6408–6414.
- Hansen, M., S. Boitano, E.R. Dirksen, and M.J. Sanderson. 1995. A role for phospholipase C activity but not ryanodine receptors in the initiation and propagation of intercellular calcium waves. *J. Cell Sci.* 108(Pt 7):2583–2590.
- Hermoso, M., N. Barrera, B. Morales, S. Perez, and M. Villalon. 2001. Platelet activating factor increases ciliary activity in the hamster oviduct through epithelial production of prostaglandin E2. *Pflugers Arch.* 442:336–345.
- Hoffmann, E.K., and P.B. Dunham. 1995. Membrane mechanisms and intracellular signalling in cell volume regulation. *Int. Rev. Cytol.* 161:173–262.
- Homolya, L., T.H. Steinberg, and R.C. Boucher. 2000. Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia. *J. Cell Biol.* 150:1349–1360.
- Houtmeyers, E., R. Gosselink, G. Gayan-Ramirez, and M. Decramer. 1999. Regulation of mucociliary clearance in health and disease. *Eur. Respir. J.* 13:1177–1188.
- Kiselyov, K., J.Y. Kim, W. Zeng, and S. Muallem. 2005. Protein-protein interaction and function of TRPC channels. *Pflugers Arch.* 451:116–124.
- Lansley, A.B., and M.J. Sanderson. 1999. Regulation of airway ciliary activity by Ca^{2+} : simultaneous measurement of beat frequency and intracellular Ca^{2+} . *Biophys. J.* 77:629–638.
- Lehtonen, J.Y., and P.K. Kinnunen. 1995. Phospholipase A2 as a mechanosensor. *Biophys. J.* 68:1888–1894.
- Li, Y., J.M. Wright, F. Qian, G.G. Germino, and W.B. Guggino. 2005. Polycystin 2 interacts with type I inositol 1,4,5-trisphosphate receptor to modulate intracellular Ca^{2+} signaling. *J. Biol. Chem.* 280:41298–41306.
- Liedtke, W., Y. Choe, M.A. Marti-Renom, A.M. Bell, C.S. Denis, A. Sali, A.J. Hudspeth, J.M. Friedman, and S. Heller. 2000. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell.* 103:525–535.
- Liedtke, W., D.M. Tobin, C.I. Bargmann, and J.M. Friedman. 2003. Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA.* 100:14531–14536.
- Lock, H., and M.A. Valverde. 2000. Contribution of the Isk (MinK) potassium channel subunit to regulatory volume decrease in murine tracheal epithelial cells. *J. Biol. Chem.* 275:34849–34852.
- McCarty, M.A., and R.G. O'Neil. 1992. Calcium signalling in cell volume regulation. *Physiol. Rev.* 72:1037–1061.
- Montell, C. 2005. The TRP superfamily of cation channels. *Sci. STKE.* 2005:re3.
- Moore, A.L., M.W. Roe, R.F. Melnick, and S.D. Lidofsky. 2002. Calcium mobilization evoked by hepatocellular swelling is linked to activation of phospholipase C γ . *J. Biol. Chem.* 277:34030–34035.
- Morales, B., N. Barrera, P. Uribe, C. Mora, and M. Villalon. 2000. Functional cross talk after activation of P2 and P1 receptors in oviductal ciliated cells. *Am. J. Physiol. Cell Physiol.* 279:C658–C669.
- Okada, S.F., R.A. Nicholas, S.M. Kreda, E.R. Lazarowski, and R.C. Boucher. 2006. Physiological regulation of ATP release at the apical surface of human airway epithelia. *J. Biol. Chem.* 281:22992–23002.
- Pedersen, S., I.H. Lambert, S.M. Thoroed, and E.K. Hoffmann. 2000. Hypotonic cell swelling induces translocation of the alpha isoform of cytosolic phospholipase A2 but not the gamma isoform in Ehrlich ascites tumor cells. *Eur. J. Biochem.* 267:5531–5539.
- Puchelle, E., J.M. Zahm, and D. Quemada. 1987. Rheological properties controlling mucociliary frequency and respiratory mucus transport. *Biorheology.* 24:557–563.
- Sachs, F., and C.E. Morris. 1998. Mechanosensitive ion channels in nonspecialized cells. *Rev. Physiol. Biochem. Pharmacol.* 132:1–77.
- Salathe, M. 2007. Regulation of mammalian ciliary beating. *Annu. Rev. Physiol.* 69:401–422.
- Sanderson, M.J., and E.R. Dirksen. 1986. Mechanosensitivity of cultured ciliated cells from the mammalian respiratory tract: implications for the regulation of mucociliary transport. *Proc. Natl. Acad. Sci. USA.* 83:7302–7306.
- Satir, P., and M.A. Sleight. 1990. The physiology of cilia and mucociliary interactions. *Annu. Rev. Physiol.* 52:137–155.
- Shuttleworth, T.J. 1999. What drives calcium entry during $[\text{Ca}^{2+}]_i$ oscillations? challenging the capacitative model. *Cell Calcium.* 25:237–246.
- Spungin, B., and A. Silberberg. 1984. Stimulation of mucus secretion, ciliary activity, and transport in frog palate epithelium. *Am. J. Physiol.* 247:C299–C308.
- Strotmann, R., C. Harteneck, K. Nunnenmacher, G. Schultz, and T.D. Plant. 2000. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat. Cell Biol.* 2:695–702.
- Strotmann, R., G. Schultz, and T.D. Plant. 2003. Ca^{2+} -dependent potentiation of the nonselective cation channel TRPV4 is mediated by a C-terminal calmodulin binding site. *J. Biol. Chem.* 278:26541–26549.
- Suzuki, M., K. Kawahara, A. Ogawa, T. Morita, Y. Kawaguchi, S. Kurihara, and O. Sakai. 1990. $[\text{Ca}^{2+}]_i$ rises via G protein during regulatory volume decrease in rabbit proximal tubule cells. *Am. J. Physiol.* 258:F690–F696.
- Suzuki, M., A. Mizuno, K. Kodaira, and M. Imai. 2003. Impaired pressure sensation in mice lacking TRPV4. *J. Biol. Chem.* 278:22664–22668.
- Tamm, S. 1994. Ca^{2+} channels and signalling in cilia and flagella. *Trends Cell Biol.* 4:305–310.
- Teilmann, S.C., A.G. Byskov, P.A. Pedersen, D.N. Wheatley, G.J. Pazour, and S.T. Christensen. 2005. Localization of transient receptor potential ion channels in primary and motile cilia of the female murine reproductive organs. *Mol. Reprod. Dev.* 71:444–452.
- Tovey, S.C., S.P. De, P. Lipp, D. Thomas, K.W. Young, L. Missiaen, S.H. De, J.B. Parys, M.J. Berridge, J. Thuring, et al. 2001. Calcium puffs are generic InsP(3)-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. *J. Cell Sci.* 114:3979–3989.
- Tuvia, S., A. Almagor, A. Bitler, S. Levin, R. Korenstein, and S. Yedgar. 1997. Cell membrane fluctuations are regulated by medium macroviscosity: evidence for a metabolic driving force. *Proc. Natl. Acad. Sci. USA.* 94:5045–5049.
- Vandenburgh, H.H., J. Shansky, P. Karlisch, and R.L. Solerssi. 1993. Mechanical stimulation of skeletal muscle generates lipid-related second messengers by phospholipase activation. *J. Cell. Physiol.* 155:63–71.
- Vriens, J., H. Watanabe, A. Janssens, G. Droogmans, T. Voets, and B. Nilius. 2004. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc. Natl. Acad. Sci. USA.* 101:396–401.

- Watanabe, H., J.B. Davis, D. Smart, J.C. Jerman, G.D. Smith, P. Hayes, J. Vriens, W. Cairns, U. Wissenbach, J. Prenen, et al. 2002a. Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. *J. Biol. Chem.* 277:13569–13577.
- Watanabe, H., J. Vriens, S.H. Suh, C.D. Benham, G. Droogmans, and B. Nilius. 2002b. Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. *J. Biol. Chem.* 277:47044–47051.
- Watanabe, H., J. Vriens, J. Prenen, G. Droogmans, T. Voets, and B. Nilius. 2003. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature*. 424:434–438.
- Winters, S.L., C.W. Davis, and R.C. Boucher. 2007. Mechanosensitivity of mouse tracheal ciliary beat frequency: roles for Ca^{2+} , purinergic signaling, tonicity, and viscosity. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 292:L614–L624.
- Wissenbach, U., M. Boddling, M. Freichel, and V. Flockerzi. 2000. Trp12, a novel Trp related protein from kidney. *FEBS Lett.* 485:127–134.
- Xu, F., E. Satoh, and T. Iijima. 2003. Protein kinase C-mediated Ca^{2+} entry in HEK 293 cells transiently expressing human TRPV4. *Br. J. Pharmacol.* 140:413–421.
- Yule, D.I., and D.V. Gallacher. 1988. Oscillations of cytosolic calcium in single pancreatic acinar cells stimulated by acetylcholine. *FEBS Lett.* 239:358–362.
- Zholos, A., B. Beck, V. Sydorenko, L. Lemonnier, P. Bordat, N. Prevarskaya, and R. Skryma. 2005. Ca^{2+} - and volume-sensitive chloride currents are differentially regulated by agonists and store-operated Ca^{2+} entry. *J. Gen. Physiol.* 125:197–211.