Phosphatidylinositol-4,5-Biphosphate-Dependent Rearrangement of TRPV4 Cytosolic Tails Enables Channel Activation by Physiological Stimuli

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1073/pnas.1220231110

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:12563734

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
PIP$_2$-DEPENDENT REARRANGEMENT OF TRPV4 CYTOSOLIC TAILS ENABLES CHANNEL ACTIVATION BY PHYSIOLOGICAL STIMULI

Anna Garcia-Elias$^{a,1}$, Sanela Mrkonjić$^{a,1}$, Carlos Pardo-Pastor$^{a}$, Hitoshi Inada$^{b}$, Ute A. Hellmich$^{b}$, Fanny Rubio-Moscadó$^{a}$, Cristina Plata$^{a}$, Rachelle Gaudet$^{b}$, Rubén Vicente$^{a}$ and Miguel A. Valverde$^{a,2}$

$^a$Laboratory of Molecular Physiology and Channelopathies, Dept. of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain; $^b$Dept. of Molecular and Cellular Biology, Harvard University, Cambridge, USA.

Submitted to Proceedings of the National Academy of Sciences of the United States of America

Most TRP channels are regulated by phosphatidylinositol-4,5-biphosphate (PIP$_2$), although the structural rearrangements occurring upon PIP$_2$ binding are currently far from being understood. Here we report that TRPV4 activation by hypotonic and heat stimuli requires PIP$_2$ binding to and rearranging of the cytosolic tails. Neutralization of the positive charges within the sequence RKKRR in the transmembrane domains 3 and 4 (27). Analysis of disease-causing mutations modifying channel activity that lay in regions close to the channel pore or within the ankyrin repeats (28) has also contributed to our understanding of relevant protein domains.

Most TRP channels are regulated by phosphatidylinositol phosphates, particularly by phosphatidylinositol-4,5-biphosphate (PIP$_2$), which is the most abundant phosphoinositide in the inner leaflet of the plasma membrane (29, 30). In general terms, it is proposed that PIP$_2$ modulates TRP channel gating and/or the sensitivity to activating stimuli (29, 30). The interaction of PIP$_2$ with TRPs involves protein regions characterised by the presence of several positively charged residues. Mutations of these positive residues (31-33) and manipulation of the PIP$_2$ levels in intact cells (34) or in excised patches (33) have been the main tools to evaluate PIP$_2$-mediated channel regulation.

The recent report of the crystal structure of K$^+$ channels with bound PIP$_2$ provides the first atomistic description of a molecular mechanism by which PIP$_2$ regulates channel activity (35, 36). PIP$_2$ binding induces a large conformation change in the protein, expanding and bringing the cytosolic domains closer to the transmembrane domains (35). Whether PIP$_2$ modulation of TRP channels involves similar conformational changes is still an open question.

We now show that TRPV4 requires the interaction of PIP$_2$ with a stretch of positive charges at the N-tail, prior to the proline-rich domain (PRD, residues 132-144), in order to be activated by hypotonicity and heat. Moreover, we have also demonstrated that the reported lack of channel response to heat in excised patches is fully recovered in the presence of PIP$_2$, thereby suggesting that TRPV4 is bona fide thermosensitive channel. Finally, reduction of PIP$_2$ levels or disruption of the PIP$_2$ interaction with the channel increased FRET signal between fluorescent probes on the TRPV4 cytosolic tails, consistent with a more compact cytosolic region. This is the first piece of evidence suggesting that, similar to PIP$_2$-regulated K$^+$ channels, PIP$_2$ interaction with the TRPV4 channel rearranges cytosolic domains.

RESULTS AND DISCUSSION

A possible phosphoinositide interacting site in the TRPV4 N-tail is required for channel activation by hypotonicity and heat. We

Reserved for Publication Footnotes
Fig. 1. Functional analysis of N-terminal truncations and mutations of TRPV4. (A) Mean current density measured at +100 and -100 mV in response to a 30% hypotonic shock in HEK-293 cells overexpressing TRPV4-WT, TRPV4-Δ1-30, TRPV4-Δ1-130, TRPV4-Δ100-130 and GFP. Number of cells recorded is shown for each condition. (B) Ramp current-voltage relations of cationic currents recorded from HEK-293 cells transfected with TRPV4-WT or TRPV4-Δ121 AAWAA and exposed to 30% hypotonic shocks. (C) Mean current responses to isotonic and hypotonic stimuli in cells transfected with TRPV4-WT or TRPV4-Δ121 AAWAA. (D) Mean current responses to 4α-PDD stimulation in TRPV4-WT or TRPV4-Δ121 AAWAA expressing cells. (E) Calcium signals (Fura-2 ratio) obtained in HeLa cells transfected with GFP (n=47), TRPV4-WT (n=25) or TRPV4-Δ121 AAWAA (n=59) and sequentially stimulated with 30% hypotonic solutions and 10 μM 4α-PDD. (F) Calcium signals obtained in HeLa cells transfected with TRPV4-WT (n=335), TRPV4-Δ121 AAWAA (n=318) or GFP (n=256) and stimulated with warm solutions (38°C). * P<0.05.

Fig. 2. Effect of PIP2 depletion on TRPV4-mediated Ca2+ signals. HeLa cells were transfected with TRPV4-WT, TRPV4-WT plus FRB and FKBP5-PHOS-phosphatase (PIP2-Ptds) or the inactive phosphatase (D281A). (A-C) Average calcium signals (fura-2 ratio) measured in the presence of the phosphatase translocation inducing agent rapamycin (1 μM) for cells exposed to (A) 30% hypotonicity (TRPV4, n=302; V4+PIP2-Ptds, n=229; V4+PIP2-Ptds-D281A, n=192), (B) heat (TRPV4, n=150; V4+PIP2-Ptds, n=146; V4+PIP2-Ptds-D281A, n=89) and (C) 4α-PDD (TRPV4, n=278; V4+PIP2-Ptds, n=233; V4+PIP2-Ptds-D281A, n=89). (D-G) Representative intracellular Ca2+ signals obtained from mouse tracheal ciliated cells exposed to a hypotonic solution in the absence (D) or the presence (E) of 20 μM ATP, or exposed to heat (38°C) in the absence (F) or the presence (G) of 20 μM ATP. Percentage of ciliated cells responding to hypotonicity and heat was >90%. In the presence of ATP the percentages were <5% (hypotonicity) and >90% (heat).
significant increases in intracellular Ca\(^{2+}\) levels in cells expressing TRPV4-WT while cells expressing TRPV4-121AAWAA only responded to 4α-PDD (Fig. 1E).

TRPV4 is also activated by moderate heat (above 25ºC) (8, 21), although the mechanism of its temperature sensitivity is not fully understood (8). Ca\(^{2+}\) imaging on cells exposed to warm temperatures (38ºC) revealed a typical transient response in cells transfected with TRPV4-WT channels. Neutralization of the positive charges in the TRPV4-121AAWAA decreased the Ca\(^{2+}\) response to the levels obtained in GFP-transfected cells (Fig. 1F).

We hypothesized that the sequence 121KRWRK\(^{125}\) may form a PI-site required for phosphatidylinositol-4,5-biphosphate (PIP\(_2\)) interaction with TRPV4 to respond to hypotonic and heat stimuli. For that purpose, we evaluated the phosphoinositide specificity of TRPV4 activation by hypotonicity and heat. We used purified protein corresponding to residues 1-397 of human TRPV4 (150 kDa) and TRPV4-WT with phosphatidylinositol-4,5-biphosphate (PIP\(_2\)) binding to the TRPV4 N-tail. (A), Coomassie-stained SDS-PAGE showing protection from limited papain digestion by PIP\(_2\) but not PI. Purified protein corresponding to residues 1-397 of human TRPV4 (150 kDa) was digested with papain (38 nM) in the absence or presence of lipid (PI and PIP\(_2\)) at 10 µM. The cleavage positions corresponding to each isolated band, determined by N-terminal sequencing, are indicated. (B-E) The four indicated bands were scanned, quantified and plotted versus digestion time. Significant changes were observed in the presence of PIP\(_2\) for bands 1 and 3 at all times while band 4 showed significant differences at time 45 and 60 min. Mean±S.D. (n=3). * P<0.05 control vs PI; ** P<0.01 control and PI vs PIP\(_2\).

**Depletion of PIP\(_2\) levels prevents channel activation by physiological stimuli.** We assessed whether deletion or mutation of residues 121KRWRK\(^{125}\) may be related to a PIP\(_2\) mode of TRPV4 gating. For that purpose, we evaluated the impact of reducing PIP\(_2\) levels on channel activation. We used a rapamycin-induced translocatable 5-phosphatase to deplete PIP\(_2\) (39). The membrane-localized rapamycin-binding protein FRB and the cytosolic enzyme construct FKBP-5-phosphatase were co-transfected with TRPV4-WT in HeLa cells. Addition of
ical oscillatory Ca signals generated by hypotonic solutions (Fig. 2F-G). The reduction in hypotonicity- and heat-induced signal were not due to Ca2+-dependent rearrangement of TRPV4 cytosolic tails. (Fig. 2A). Application of rapamycin to cells co-transfected with TRPV4 and a phosphatase-dead mutant (Δ1-130, or CFP- and YFP-fused TRPV4-WT coexpressed with either PACSIN3 or PACSIN3-ΔF-BAR) demonstrated using 4μM 121 or with the TRPV4 blocker HC-067047 (42) or the TRPV4-ΔN region (residues 1-397), which includes the N-terminal tail and the ankyrin repeats. Papain digestion led to cleavage at four positions penetrant and remodel the plasma membrane (45,46). PACSIN3 belongs to a family of proteins that contain a Bin-Amphiphysin-Rvs (BAR) domain required to penetrate and remodel the plasma membrane (45, 46). PACSIN3 binds through its SRC homology 3 (SH3) domain to the PRD of TRPV4 (44), in the close proximity of the PRD-site. Two competing hypotheses are that membrane-bound PACSIN3 binding to the PRD may either promote or physically block the interaction of PIP2-regulated channels typically decreases in excised inside-out patches and recovers upon addition of exogenous PIP2 (40). In those excised patches in which TRPV4 channel activity was present immediately after excision, channel activity decreased with time and addition of the water soluble diC8-PIP2 (50-200 μM) or long acyl chain PIP2 (10 μM) did not recover initial channel activity (Fig. 3A and C). The fact that PIP2 was not able to activate TRPV4 in excised patches may indicate that following patch excision another, yet unidentified, modulator required for channel activity is lost. Hypotonicity-mediated activation of TRPV4 in excised patches can not be directly evaluated. Instead, the osmotransducing cytosolic messenger 5'-epoxyeicosaatrienoic acid (EET) has been used (20,41). Addition of EET (1 μM) in the presence of PIP2 activated TRPV4 in 71% of patches (Fig. 3A and C). However, addition of EET in the absence of PIP2 only activated 20% of patches (Fig. 3B and C), even though TRPV4 channel activity in the same patches was demonstrated using 4μM-PDD (Fig. 3B).

Next, we tested channel activation by heat in excised inside-out patches obtained from HeLa cells overexpressing TRPV4. In the presence of PIP2 TRPV4-WT channel activity was detected within seconds after application of warm solutions (Fig. 3D) while in the presence of PIP2 and the TRPV4 blocker HC-067047 (42) or with the TRPV4-ΔN only activated 20% of patches (Fig. 3B and C), revealing a shear-stress dependent component under our experimental conditions for heat activation of TRPV4 (Fig. 3F). In the absence of PIP2, and consistent with previous reports (8, 21, 43), no significant change in channel activity was elicited by heat (Fig. 3E). Fig. 3F shows mean channel activity in response to heat and plotted versus time after addition of warm solutions in the presence or absence of PIP2. The TRPV4 Q12 obtained from excised patches containing TRPV4-WT in the presence of PIP2 was 21±5 (n=3) (Fig. 3B), consistent with previous values obtained from TRPV4 whole-cell recordings (21, 43). Together these experiments confirm that PIP2 is required for TRPV4 activation by physiological stimuli, probably acting as an allosteric modulator. However, at present we do not have a comprehensive model to incorporate all factors involved in TRPV4 gating, i.e., why TRPV4 gating by 4μM-PDD is not affected by PIP2 depletion or why PIP2 is unable to activate TRPV4 on its own. TRPV4 interacts with the TRPV4 N-tail. To further characterize PIP2 interaction with the TRPV4 N-tail, we carried out limited proteolysis assays on the purified TRPV4 N-terminal region (residues 1-397), which includes the N-terminal tail and the ankyrin repeats. Papain digestion led to cleavage at four positions within the N-tail (Fig. 4A). Quantification of the bands obtained from the proteolysis of TRPV4 N-terminal region (residues 1-397), which includes the N-terminal tail and the ankyrin repeats (Fig. 4B-E) showed that proteolysis of TRPV4 N-terminus is decreased with time and addition of the water soluble diC8-PIP2 (10 μM) did not recover initial channel activity (Fig. 3A and C). The fact that PIP2 was not able to activate TRPV4 in excised patches may indicate that following patch excision another, yet unidentified, modulator required for channel activity is lost. Hypotonicity-mediated activation of TRPV4 in excised patches can not be directly evaluated. Instead, the osmotransducing cytosolic messenger 5'-epoxyeicosaatrienoic acid (EET) has been used (20,41). Addition of EET (1 μM) in the presence of PIP2 activated TRPV4 in 71% of patches (Fig. 3A and C). However, addition of EET in the absence of PIP2 only activated 20% of patches (Fig. 3B and C), even though TRPV4 channel activity in the same patches was demonstrated using 4μM-PDD (Fig. 3B).
with the interaction of TRPV4 with PIP₂ is therefore consistent with the hypothesis that PACSIN3 interferes with the whole-cell currents generated by hypotonic challenges (Fig. 5A). The channel response to 4α-PDD was not affected under any of the experimental conditions tested (Fig. 5B). These results were therefore consistent with the hypothesis that PACSIN3 interferes with the interaction of TRPV4 with PIP₂, an effect that was lost when a membrane-unbound PACSIN3-ΔF-BAR was used.

**PIP₂ rearranges TRPV4 cytosolic tails.** Together, our findings underscore the involvement of PIP₂ in TRPV4 gating by physiological stimuli. However, an important question remained that has not been resolved for any PIP₂-modulated TRP channel yet. Does PIP₂ binding affect the structural conformation of TRPV4? We approached this question studying the impact of TRPV4 deletions and mutations on the conformation of cytosolic tails. For that purpose we evaluated the proximity of the intracellular C-tails of CFP- and YFP-tagged TRPV4 proteins, which we assumed formed a random population of heterogeneous channels, by fluorescence resonance energy transfer (FRET). We tagged C-tails, which remained unmodified in all the TRPV4 deletions/mutations generated, to avoid possible FRET artifacts generated by the different lengths of the N-tails. The relative CFP and YFP fluorescence intensities in the plasma membrane rearranged the cytosolic TRPV4 tails into a more compacted form (Fig. 5C). TRPV4-WT generated a FRET ratio similar to that previously reported (48) while TRPV4-Δ1-130 and TRPV4-ΔΔF-BAR were unmodified in all the TRPV4 deletions/mutations generated, to avoid possible FRET artifacts generated by the different lengths of the N-tails. The relative CFP and YFP fluorescence intensities in the plasma membrane were determined for every single cell used to calculate FRET efficiencies in transiently transfected HEK-293 cells (Fig. 5C).

**PIP₂ modulation by TRPV4.** We overexpressed CFP- and YFP-tagged TRPV4-WT channels in HEK293 cells engineered with tetracycline-inducible expression of 5-phosphatase IV (33). Induction of this phosphatase provided the first piece of evidence suggesting that, similar to PIP₂-regulated K⁺ channels, PIP₂ interaction with TRPV4 channel rearranges the cytosolic domains. Whether the intracellular tail rearrangement occurring upon PIP₂ binding to TRPV4 facilitates the access of stimuli-generated messengers (e.g., EET) to their binding sites or favors the stimulus-dependent opening of the gates themselves it is not known at present.

**MATERIALS AND METHODS**

**Cells and transfection.**

For electrophysiological or calcium imaging experiments HeLa or HEK-293 cells were transiently transfected as previously described (16, 48). Primary cultures of tracheal epithelial cells were obtained as previously described (7, 17). Animals were maintained and experiments were performed according to the guidelines issued by the Institutional Ethics Committee of the Universitat Pompeu Fabra.

**Solutions.**

Isotonic bath solutions used for imaging experiments contained (in mM): 140 NaCl, 2.5 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5 glucose and 10 HEPES, pH 7.3 with Tris. Bath solutions for whole-cell recordings contained (in mM): 100 NaCl, 1 MgCl₂, 6 CaCl₂, 10 HEPES, 1 EGTA and 5 glucose, pH 7.3 with Tris. Osmolarity was adjusted to 310 mosM using mannitol. 30% and 15% hypotonic solutions (255 and 220 mosM) were obtained by removing mannitol. Whole-cell pipette solution contained (in mM): 20 CsCl, 100 CsAcetate, 1 MgCl₂, 0.1EGTA, 4 HEPS, 4 NaATP, and 1 NaGTP, 300 mosM, pH 7.25. Bath and pipette solutions for inside-out single channel recordations contained (in mM): 130 CsCl, 1 MgCl₂, NaATP, 0.33CaCl₂, 5 EGTA, 10 HEPS (310 mosmolliter, pH 7.25). When required, solutions were warmed using a water jacket device (Warner Instruments). All chemicals were obtained from Sigma-Aldrich (St.Louis, MO, U.S.A.) for rapamycin-inducible phosphatases and Dr. M.Schaefer (Leipzig, Germany) for help with initial FRETexperiments. This work was supported in part by grants from the Universitat Pompeu Fabra.

**Electrophysiological and Ratiometric Ca²⁺ recordings.**

Patch-clamp whole-cell and single-channel currents were recorded at room temperature (−25°C, unless otherwise indicated) as previously described (16, 48). Cells/excisioned patches were perfused at 0.8 mlin/min. Cytosolic Ca²⁺ signals, relative to the ratio (340/380)measured prior to stimulation, were obtained from cells loaded with 4.5 μM fura-2 AM as previously described (4).

**FRET measurements.**

FRET measurements were carried out in a Leica TCS SP2 confocal microscope (Leica) attached to an inverted microscope. FRET efficiencies were expressed as the increase of the FRET donor CFP after bleaching the FRET acceptor YFP (48).

**Lipid protection assay.**

Human TRPV4 ankyrin repeats (136-397) and N-tail (1-397) were cloned using Ndel and Noti into pET21-C6H (49). Recombinant proteins were produced and purified as described (50), except the size exclusion chromatography buffer was 10 mM Tris-HCl pH 7.0, 300 mM NaCl, 10 % glycerol, and 1 mM DTT for TRPV4ankyrin repeats. Lipid protection assay by limited proteolysis was performed at 4°C (on ice) in reaction buffer containing (in mM): 180 NaCl, 20 Tris-HCI pH 7.0, 1 % glycerol and 1 DTT (for TRPV4-1-397) or 150 NaCl, 20 Tr-HCI pH 7.0 and 1 DTT (for TRPV4-136-397 and TRPV4-ARD). Proteins were pre-incubated in the absence or presence of PI or PIP₂, at 4°C for 60 min and then digested with papain. Final concentrations of protein, lipid, and papain were 10 μM, 150 mM and 0.25 mg/ml respectively. Digestion was observed by adding SDS sample buffer, and samples separated by SDS-PAGE and visualized by Coomassie staining. The gels were scanned and signals were quantified with ImageJ.

Data are expressed as means±SEM (or means±S.D. in Fig. 4) of n of experi-

ments. Statistical analysis was assessed with Student’s unpaired test or one-way analysis of variance (ANOVA) using Sigma-Plot software.

**Acknowledgments.**

We thank Dr T. Voets (KU Leuven, Belgium) for the gift of HEK-293 transfectant inducible phosphatase expressing cells, Dr. T. Meyer (Stanford, U.S.A.) for rapamycin-inducible phosphatases and Dr. M. Schaefer (Leipzig, Germany) for help with initial FRETEXperiments. This work was supported by the Spanish Ministry of Science and Innovation (SAF2012-38140; Fondo de Investigacion Sanitaria (Red HERACLES RD12/0042/0014); FEDER Funds; Generalitat de Catalunya (SGR05-266); and National Institutes of Health (1R01GM083592). M.A.V. is a recipient of an EMBO Long-Term Fellowship. Footnotes *A-G.E. and S.M. contributed equally to this work. **To whom correspondence should be addressed. ***Miguel.valverde@upf.edu Author contributions: A.G.-E., F.R.-M., R.V., and M.A.V. designed research; A.G.-E., S.M., C.P.-P., H.I., F.R.-M., C.P., U.A.H. and R.V. performed research; A.G.-E., S.M., C.P.-P., H.I., U.A.H. and M.A.V. analyzed data; and M.A.V. wrote the paper. All authors collaborated in paper edition. The authors declare no conflict of interest. This article contains supporting information online.


Please review all the figures in this paginated PDF and check if the figure size is appropriate to allow reading of the text in the figure.

If readability needs to be improved then resize the figure again in 'Figure sizing' interface of Article Sizing Tool.
Figure S1. Schematic representation of the TRPV4 channel with the deletions and mutations used in this study. PRD, Proline Rich Domain. ANK, ankyrin repeats.
Figure S2. Functional analysis of N-terminal truncations of TRPV4. (A) Ramp current-voltage relations of cationic currents recorded from HEK-293 cells transfected with TRPV4-WT or GFP and exposed to isotonic and 30% hypotonic solutions. (B) Ramp current-voltage relations of cationic currents recorded from HEK-293 cells transfected with TRPV4-WT, TRPV4-Δ1-30, TRPV4-Δ1-130, TRPV4-Δ100-130 and exposed to 30% hypotonic solutions. Representative traces in (A-B) correspond to the average values shown in Fig. 1A of the main text. (C) Calcium signals (fura-2 ratio) obtained in HeLa cells transfected with TRPV4-WT (n=50) or TRPV4-Δ100-130 (n=70) and GFP (n=37) exposed to 30% hypotonic solutions. All cells analyzed were included. (D) Mean current density measured at +100 and -100 mV recorded from cells transfected with TRPV4-WT, TRPV4-Δ1-30 and TRPV4-Δ1-130 exposed to 15% hypotonic solutions Number of cells recorded is shown for each condition. Mean±S.E.M. * P<0.05, one way ANOVA and Bonferroni post hoc.
**Figure S3. Membrane localization of TRPV4-WT and TRPV4-Δ1-130.** (A), Confocal immunofluorescence images of non-permeabilized (left) and permeabilized (right) HEK-293 cells overexpressing TRPV4-WT tagged with V5 in the first extracellular loop. (B), Confocal immunofluorescence images of non-permeabilized (left) and permeabilized (right) HEK-293 cells overexpressing TRPV4-Δ1-130 tagged with V5 in the first extracellular loop. (C), Quantification of surface expression (normalized to total cell expression) of V5 tagged TRPV4-WT (n=9) and TRPV4-Δ1-130 (n=9) using an HRP-linked secondary antibody and chemiluminescence analysis.
Figure S4. Functional analysis of TRPV4-114 AAA. (A) Calcium signals (fura-2 ratio) obtained in HeLa cells transfected with TRPV4-114 AAA and sequentially stimulated with 30% hypotonic solutions and heat (38°C). Mean±S.E.M., n=133. (B) Peak calcium signals measured in HeLa cells cotransfected with TRPV4-WT, FRB and FKBP-5-phosphatase (PIP2-pterase) and exposed to 100 nM 4α-PDD. Cells were pretreated with the phosphatase translocation inducing agent rapamycin (1 μM). TRPV4 (n=43), TRPV4 +PIP2-Phase (n=46).
Figure S5. Translocation PH-PLC. (A-C) Confocal images of HEK cells expressing a yellow fluorescent protein (YFP)-tagged pleckstrin homology (PH) domain from phospholipase C-δ1 (PLC-δ1) serving as a PIP2 biosensor (YFP-PH(PLC-δ1)), the rapamycin-binding protein FRB and either the cytoplasmic enzyme construct FKBP-5-phosphatase (PIP2-PTase) or the inactive phosphatase (D281A). Images were taken in the absence (left, mainly plasma membrane signal) or presence (right, cytosolic signal) of the phosphatase translocation inducing agent rapamycin (1 μM). (D), Depletion of PIP2 in HEK-293 cells overexpressing a tetracycline-induced 5-phosphatase. Left, cells without treatment with tetracycline (no 5-phosphatase induction). Right, images obtained 24h after induction of 5-phosphatase with 0.5 μg/mL tetracycline.
Figure S6. Ciliated epithelial cells response to hypotonic cell swelling and heat. (A-B) Representative traces of the intracellular calcium signals obtained from mouse tracheal ciliated cells exposed to two consecutive hypotonic solutions (A) or heat stimuli (B). (C-D) Representative traces of the intracellular calcium signals obtained from mouse oviductal ciliated cells exposed to hypotonic solutions in the absence (C) or in the presence (D) of previous activation of G-protein-coupled purinergic receptors with ATP (20 μM). Note the almost complete absence of response to hypotonicity following exposure to ATP. (E) Second, within mins, stimulation with ATP did not trigger intracellular calcium signals in mouse oviductal ciliated cells.
Figure S7. Heat activation of TRPV4 channel in inside-out patches. (A) Activation of TRPV4 channel in the presence of PIP2 was not triggered by the shear stress generated by the flow of the external solution, but by the increase in temperature. (B) To calculate the $Q_{10}$ of the TRPV4 channel the same excised patch was first exposed to 28°C and then to 38°C.
Figure S8. Differential proteolysis protection of the TRPV4 N-terminus by different phosphoinositides and dependence on the 121KRWRK125 motif. (A) Analysis of PIP2-mediated proteolysis protection of the human TRPV4 N-tail lacking residues 1-135. Coomassie-stained SDS-PAGE of TRPV4 samples after 15, 30, 45 and 60 min digestion with papain in the absence or presence of PI or PIP2. (B) Analysis of PIP2-mediated proteolysis protection of the rat TRPV1 ankyrin repeats (residues 101-364). Coomassie-stained SDS-PAGE of TRPV1 samples obtained after 15, 30, 45 and 60 min digestion with papain in the absence or presence of PI or PIP2. (C), Coomassie-stained SDS-PAGE of TRPV4-121AAWAA N-tail samples obtained after 15, 30 and 45 min digestion with papain in the absence or presence of PI or PIP2, showing that none of the phosphoinositides protect the mutant from proteolysis. These gels are representative results from four similar experiments.
Figure S9. Interaction of TRPV4 with PACSIN3. FRET ratios, represented as the CFP increase during YFP photobleaching normalized to the initial CFP value, determined at the plasma membrane of HEK-293 cells expressing TRPV4-WT-CFP and either PACSIN3-YFP, PACSIN3-ΔF-BAR or soluble YFP. Number of cells recorded is shown for each condition. Mean±S.E.M. * P<0.05, one way ANOVA and Bonferroni post hoc.