PIP2-DEPENDENT REARRANGEMENT OF TRPV4 CYTOSOLIC TAILS ENABLES CHANNEL ACTIVATION BY PHYSIOLOGICAL STIMULI

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Most TRP channels are regulated by phosphatidylinositol-4,5-biphosphate (PIP2), although the structural rearrangements occurring upon PIP2 binding are currently far from being understood. Here we report that TRPV4 activation by hypotonic and heat stimuli requires PIP2 binding to and rearranging of the cytosolic tails. Neutralization of the positive charges within the sequence KRWRK, which resembles a phosphoinositide binding site, rendered the channel unresponsive to hypotonicity and heat but directly to 4α-phorbol 12,13-didecanoate and abolished binding to and transmembrane domain. Similar channel response was obtained by depleting PIP2 from the plasma membrane with translocatable phosphatases in heterologous expression systems or by activation of phospholipase C in native ciliated epithelial cells. PIP2 facilitated TRPV4 activation by the osmotransducing cytosolic messenger 3-6-epoxyeicosatrienoic acid and allowed channel activation by heat in inside-out patches. Protease protection assays demonstrated a PIP2 binding site within the N-tail. The proximity of TRPV4 tails, analysed by fluorescence resonance energy transfer, increased by depleting PIP2, mutations in the PI-site or by co-expression with PACSIN3, a regulatory molecule that binds TRPV4 N-tails and abrogates activation by cell swelling and heat. PACSIN3 lacking the F-BAR domain interacted with TRPV4 without affecting channel activation or tail rearrangement. Therefore, mutations weakening the TRPV4-PIP2 interacting site and conditions that deplete PIP2 or restrict TRPV4 access to PIP2—in the case of PACSIN3—change tail conformation and negatively affect channel activation by hypotonicity and heat.

INTRODUCTION

TRPV4 is a non-selective cation channel that responds to osmotic (1-4), mechanical (5-7) and temperature stimulation (8, 9), thereby contributing to many different physiological functions: cellular (4, 9) and systemic volume homeostasis (10), vasodilatation (11, 12), nociception (13), epithelial hydroelectrolyte transport (14), bladder voiding (15), ciliary beat frequency regulation (7, 16), chondroprotection (18) and skeletal regulation (19). Osmotic (20) and mechanical (7, 16) sensitivity of TRPV4 depends on phospholipase A2 activation and the subsequent production of the arachidonic acid metabolites, epoxyeicosatrienoic acids (EET), while the mechanism leading to temperature-mediated activation (only observed in intact cells) it is not known at present (21). Reports also exist claiming EET-independent TRPV4 activation by membrane stretch in excised-patches from oocytes (22), in apparent contradiction with early reports claiming lack of activation by membrane stretch (1). Several studies have characterized TRPV4 domains implicated in channel regulation by calmodulin (23, 24), PACSIN3 (25), intracellular ATP (24) and inositol-trisphosphate receptor (16, 26). However, little is known about the domains relevant for TRPV4 activation by different stimuli, apart from the interaction between the TRPV4 activator 4α-phorbol 12,13-didecanoate (4α-PDD) and 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 (PIP2), which is the most abundant phosphoinositide in the inner leaflet of the plasma membrane (29, 30). In general terms, it is proposed that PIP2 modulates TRP channel gating and/or the sensitivity to activating stimuli (29, 30). The interaction of PIP2 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 PIP2 levels in intact cells (34) or in excised patches (33) have been the main tools to evaluate PIP2-mediated channel regulation.

The recent report of the crystal structure of K+ channels with bound PIP2 provides the first atomistic description of a molecular mechanism by which PIP2 regulates channel activity (35, 36). PIP2 binding induces a large conformation change in the protein, expanding and bringing the cytosolic domains closer to the transmembrane domains (35). Whether PIP2 modulation of TRP channels involves similar conformational changes is still an open question.

We now show that TRPV4 requires the interaction of PIP2 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 PIP2, thereby suggesting that TRPV4 is bona fide thermosensitive channel. Finally, reduction of PIP2 levels or disruption of the PIP2 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 PIP2-regulated K+ channels, PIP2 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

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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-Δ121AAWAA and exposed to 30% hypotonic shock. (C) Mean current response to isotonic and hypotonic stimuli in cells transfected with TRPV4-WT or TRPV4-Δ121AAWAA. (D) Mean current responses to 4α-PDD stimulation in TRPV4-WT or TRPV4-Δ121AAWAA expressing cells. (E) Calcium signals (Fura-2 ratio) obtained in HeLa cells transfected with GFP (n=47), TRPV4-WT (n=25) or TRPV4-Δ121AAWAA (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-Δ121AAWAA (n=318) or GFP (n=254) 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 FKBP-5-PHIP2-phosphatase (PIP2-Ph) 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-Ptase, n=229; V4+PIP2-PtaseD281A, n=192), (B) heat (TRPV4, n=150; V4+PIP2-Ptase, n=146; V4+PIP2-PtaseD281A, n=89) and (C) 4α-PDD (TRPV4, n=278; V4+PIP2-Ptase, n=223; V4+PIP2-PtaseD281A, 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\textsuperscript{2+} levels in cells expressing TRPV4-WT while cells expressing TRPV4-121AAWAA only responded to 4\mu M-PDD (Fig. 1E).

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

We hypothesized that the sequence KRWRK125 may form a PI-site required for phosphatidylinositol-4,5-biphosphate (PIP\textsubscript{2}) interaction with TRPV4 to respond to hypotonic and heat stimuli. Different TRP protein sequences containing several positively charged amino acids have been proposed to interact with phosphoinositides, particularly PIP\textsubscript{2} (29, 38). To examine how specific was the neutralization of the KRWRK125 positive charges, we neutralized three positive charges of a nearby region (RHH). Expression of TRPV4-125AAA produced hypotonicity and heat-induced Ca\textsuperscript{2+} increases similar to those obtained with TRPV4-WT (Fig S4A). Together, these experiments suggested that residues KRWRK125 are critical for TRPV4 activation by hypotonicity and heat, but not necessary for channel activation by 4\mu M-PDD.

Depletion of PIP\textsubscript{2} levels prevents channel activation by physiological stimuli. We assessed whether deletion or mutation of residues KRWRK125 may be related to a PIP\textsubscript{2} dependent mode of TRPV4 gating. For that purpose, we evaluated the impact of reducing PIP\textsubscript{2} levels on channel activation. We used a rapamycin-induced translocatable 5-phosphatase to deplete PIP\textsubscript{2} (39). The membrane-localized rapamycin-binding protein FRB and the cytoplasmic enzyme construct FKBP-S-phosphatase were co-transfected with TRPV4-WT in HeLa cells. Addition of

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Activity of PIP$_2$-regulated channels typically decreases in excised inside-out patches and recoveries upon addition of exogenous PIP$_2$ (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-PIP$_2$ (50-200 μM) or long acyl chain PIP$_2$ (10 μM) did not recover initial channel activity (Fig. 3A and C). The fact that PIP$_2$ 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. Hypoosmotic-mediated activation of TRPV4 in excised patches can not be directly evaluated. Instead, the osmoto-sensing cytosolic messenger 5'-G-epoxyeicosatrienoic acid (EET) has been used (20,41). Addition of EET (1 μM) in the presence of PIP$_2$ activated TRPV4 in 71% of patches (Fig. 3A and C). However, addition of EET in the absence of PIP$_2$ only activated 20% of patches (Fig. 3B and C), even though TRPV4 channel activity in the same patches was demonstrated using 40-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 PIP$_2$, TRPV4-WT channel activity was detected within seconds after application of warm solutions (Fig. 3D) while in the presence of PIP$_2$, and the TRPV4 blocker HC-067047 (42) or with the TRPV4-123 AA/AWAA no channel activity was elicited by heat (Fig. 3F). We discarded a shear-stress dependent component under our experimental conditions for heat activation of TRPV4 (Fig. S7A). In the absence of PIP$_2$, 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 PIP$_2$. The TRPV4 Q$_{20}$ obtained from excised patches containing TRPV4-WT in the presence of PIP$_2$ was 21±5 (n=3) (Fig. S7B), consistent with previous values obtained from TRPV4 whole-cell recordings (21, 43). Together these experiments confirm that PIP$_2$ 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 40-PDD is not affected by PIP$_2$ depletion or why PIP$_2$ is unable to activate TRPV4 on its own.

**PIP$_2$ interacts with the TRPV4 N-tail.** To further characterize PIP$_2$ 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 (Fig. 4B-E) showed that proteolysis of TRPV4 N-terminus is reduced in the presence of PIP$_2$ but not PI. PIP$_2$-dependent proteolysis protection was not observed with the isolated TRPV4 ankyrin repeats (residues 136-397), the TRPV1 ankyrin repeats or the TRPV4-123 AA/AWAA N-terminal region (Fig. S8), ruling out non-specific inhibition of papain by PIP$_2$. These biochemical data therefore support a direct interaction of PIP$_2$ with the N-tail region of TRPV4-WT.

**PACSIN3 F-BAR domain is required for TRPV4 channel regulation.** The effect of neutralizing the positively charged residues, or depleting PIP$_2$ levels, on channel activity resembled the response of TRPV4 when coexpressed with PACSIN3, i.e., reduced channel response to hypoosmotic and heat but normal response to 40-PDD (25, 44). PACSIN3 binds through its SRC homology 3 (SH3) domain to the PRD of TRPV4 (44), in the close proximity of the PI-site. Two competing hypotheses are that membrane-bound PACSIN3 binding to the PRD may either promote or physically block the interaction...
of the PI-site with membrane PIP$_2$. To test these hypotheses, we generated a PACSIN3 lacking the F-BAR domain. Similar deletion in PACSIN1 renders the protein unable to interact with the lipids of the plasma membrane (47). The F-BAR domain of PACSIN3 is not required for interaction with TRPV4 (44). Accordingly, we detected interaction of PACSIN3-ΔF-BAR with TRPV4 (Fig. S9). Coexpression of TRPV4 with PACSIN3-ΔF-BAR, unlike coexpression with PACSIN3, did not reduce 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 interfaces with the interaction of TRPV4 with PIP$_2$, an effect that was lost when a membrane-unbound PACSIN3-ΔF-BAR was used.

PIP$_2$ rearranges TRPV4 cytosolic tails. Together, our findings underscore the involvement of PIP$_2$ in TRPV4 gating by physiological stimuli. However, an important question remained that has not been resolved for any PIP$_2$-modulated TRP channel yet. Does PIP$_2$ 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 hetero- or homodimers, 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 were determined for every single cell and used to calculate FRET efficiencies in transiently transfected HEK-293 cells (Fig. 5C).

TRPV4-WT generated a FRET ratio similar to that previously reported (48) while TRPV4-A1-130 and TRPV4-$\Delta$F-BAR$^{12}$AAWAA doubled the FRET ratio, indicating a more compacted tail conformation. Similarly, coexpression of TRPV4-WT with PACSIN3 markedly increased the FRET signal, an effect that was lost when coexpressed with PACSIN3-ΔF-BAR. We reasoned that the increased FRET observed with mutant TRPV4 proteins or when coexpressed with PACSIN3 was due to the inability of TRPV4 to interact with membrane PIP$_2$. To test this hypothesis, we studied how the reduction in PIP$_2$ levels affected FRET efficiency of TRPV4-WT. We overexpressed CFP- and YFP-tagged TRPV4-WT channels in HEK293 cells engineered with tetracycline-regulated ankryin repeats (mutations in the PI-site or coexpression with PACSIN3) or depleted PIP$_2$ by overexpression of F-BAR. We reasoned that a PI-site or by overexpression of PACSIN3) or depleted PIP$_2$ by overexpression of PACSIN3 or F-BAR with PIP$_2$, favors an expanded conformation of the intracellular tails and channel activation by hypotonicity and heat. Conditions that reduced PIP$_2$ levels (inducible phosphatase) or interfere with the interaction of F-BAR with PIP$_2$ (mutations in the PI-site or coexpression with PACSIN3) promote a compacted tail conformation and prevent channel activation by hypotonicity and heat. Our study provides the first piece of evidence suggesting that, similar to PIP$_2$-regulated K$^+$ channels, PIP$_2$ interaction with TRPV4 channel rearranges the cytosolic domains. Whether the intracellular tail rearrangement occurring upon PIP$_2$ 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 CaCl$_2$, 0.5 MgCl$_2$, 5 glucose and 10 HEPES, pH 7.3 with Tris. Bath solutions for whole-cell recordings contained (in mM): 100 NaCl, 1 MgCl$_2$, 6 Cl$_2$, 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 MgCl$_2$, 0.1EGTA, 4 HEPES, 0.1 NaATP, 0.1 MgATP, 300 mOsm, pH 7.25. Bath and pipette solutions for fluorescence assays contained (in mM): 130 CsCl, 1 MgCl$_2$, 5 NaATP, 0.34 CaCl$_2$, 5 EGTA, 10 HEPES (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) and used without further purification.

Electrophysiological and Ratiometric Ca$^{2+}$ 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/excised patches were perfused at 0.8 mlin min$. Cytosolic Ca$^{2+}$ 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 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 NdeI and NotI into pE72-T-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, 1 % glycerol, and 1 mM DTT for TRPV4-ΔF-BAR, and 10 mM Tris-HCl pH 7.0, 150 mM NaCl and 1 mM DTT for TRPV4 ankyrin repeats (R01GM081340). M.A.V. is the recipient of an ICREA Academia Award and M.A.V. analyzed data; and M.A.V. wrote the paper. All authors collaborated. E-mail: miguel.valverde@upf.edu Author contributions: A.G-E., M.A.V. and M.A.V. designed research; A.G-E., S.M., C.P.P., H.L., F.R.M., C.P., U.A.H. and R.V. performed research; M.A.G.E., S.M., C.P.P., H.L., 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.

22. Impairments of mouse bladder voiding.

19. TRPV4 channels respond directly to stretch force.

18. Functional genescreeningsystemidentifiedTRPV4asaregulator of osteoclasts.

17. TRPV4-mediatedcalciuminfluxregulatesterminaldifferentiation of epithelial ciliary activity.

15. PACSIN 3.

13. cation channel TRPV4 is mediated by a C-terminal calmodulin binding site.

10. TRPV4.

9. 5'-6'-epoxyeicosatrienoic acid.

7. In receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelial cells.

1. OTRPC4, a mammalian TRPV4 (VR-OAC) homologue.

2. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4.

3. TRPV4 cation channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelium.

4. Abnormal osmotic regulation in trpv4-/- mice.

5. Anandamide and arachidonic acid use epoxyeicosatrienoic acid to activate TRPV4 channels.

6. TRPV4 channels splice variants revealed a key role of ankyrin domains in multimerization and trafficking.

7. A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators.

8. TRPV4-mediated channelopathies.

9. Abnormal osmotic regulation in trpv4-/- mice.


11. To activate TRPV4 channels.

12. Accumulation of cholesterol is linked to TRPM8 desensitization.

13. TRPM8 channel participates in receptor-operated calcium entry.


15. TRPV2 ion channel.

16. Inhibition of the cation channel TRPV4 improves bladder function.

17. Inflammatory mediators.


19. Rab9a cytosolic phospholipase A2 regulates TRPV4 activity.

20. TRPV4 channels participate in receptor-operated calcium entry.


22. Pacsin 3.

23. Functional coupling of TRPV4 cationic channel and large conductance, calcium-dependent potassium channel in human bronchial epithelial cell lines. Pflugers Arch 457:149-159.


25. Transient receptor potential channels meet phosphoinositide regulation.


27. PtdIns(4,5)P2 signaling.


30. TRPV4.

31. TRPV4.

32. In receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelial cells.

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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 TRPV4-114AAA. (A) Calcium signals (fura-2 ratio) obtained in HeLa cells transfected with TRPV4-114AAA 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-ptase) 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 HeLa cells expressing a yellow fluorescent protein (YFP)-tagged pleckstrin homology (PH) domain from phospholipase C-δ1 (PLC-δ1) serving as a PIP_2 biosensor (YFP-PH(PLC-δ)), the rapamycin-binding protein FRB and either the cytoplasmic enzyme construct FKBP-5-phosphatase (PIP_2-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 PIP_2 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 Q₁₀ 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 $^{121}$KRWR$^{125}$ motif. (A) Analysis of PIP$_2$-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 PIP$_2$. (B) Analysis of PIP$_2$-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 PIP$_2$. (C), Coomassie-stained SDS-PAGE of TRPV4-$^{121}$AAWAA N-tail samples obtained after 15, 30 and 45 min digestion with papain in the absence or presence of PI or PIP$_2$, 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.