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

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Accessibility
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 125KRWR125, which resembles a phosphoinositol binding site, rendered the channel unresponsive to hypotonicity and heat but, directly to transmembrane domains. Similar channel response was obtained by deleting 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 5,6-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 deleting PIP2, mutations in the PI- or 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), thereby contributing to many different physiological functions: cellular (4, 9) and systemic volume homeostasis (10), vasodilatation (11, 12), nociception (13), epithelial electrolyte transport (14), bladder voiding (15), ciliary beat frequency regulation (7, 16, 17), chondroprotection (18) and skeletal regulation (19). Os- motic (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 ac- tivation 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 phosphoinositol 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 thermostensitive 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 phosphoinositol 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 transiently transfected with TRPV4-WT or TRPV4-Δ121AAWAA and exposed to 30% hypotonic shocks. (C) Mean current responses 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-phosphatase (PIP2-Pase) 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-Pase, n=229; V4+PIP2-PaseD281A, n=192), (B) heat (TRPV4, n=150; V4+PIP2-Pase, n=146; V4+PIP2-PaseD281A, n=89) and (C) 4α-PDD (TRPV4, n=278; V4+PIP2-Pase, n=223; V4+PIP2-PaseD281A, 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 25°C) (8, 21), although the mechanism of its temperature sensitivity is not fully understood (8). Ca$^{2+}$ 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-interaction with TRPV4 to respond to hypotonic and heat stimuli. We hypothesized that these sequence 121KRWRK may form a PI-site required for phosphatidylinositol-4,5-biphosphate (PIP2) binding to the TRPV4 N-tail. (A), Coomassie-stained SDS-PAGE showing protection from limited papain digestion by PIP2, 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 PIP2, 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, quantitated and plotted versus digestion time. Significant changes were observed in the presence of PIP2 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 PIP2; ** P<0.01 control and PI vs PIP2.

Depletion of PIP2 levels prevents channel activation by physiological stimuli. We assessed whether deletion or mutation of residues 121KRWRK may be related to a PIP2-dependent mode of TRPV4 gating. For that purpose, we evaluated the impact of reducing PIP2 levels on channel activation. We used a rapamycin-induced translocatable 5-phosphatase to deplete PIP2 (39). The membrane-localized rapamycin-binding protein FRB and the cytosolic enzyme construct FKBP5-phosphatase were co-transfected with TRPV4-WT in HeLa cells. Addition of

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rapamycin to translocate the phosphatase to the plasma membrane, locally depleted membrane PIP2; (Fig. SSA-C) and prevented the increase of the Ca2+ signal following hypotonic cell swelling (Fig. 2A) and heat stimulation (Fig. 2B) without affecting the response to 0.1-10 μM 4α-PDD (Fig. 2C, and Fig. S4B). Application of rapamycin to cells coexpressed with TRPV4 and a phosphatase-dead mutant (D281A) (39) did not affect the Ca2+ response to any of the stimuli tested (Fig. 2A-C).

We also analyzed whether phospholipase C ( PLC) -induced depletion of PIP2 decreased TRPV4 channel activity in native cells. For that purpose we used primary cultures of ciliated epithelial cells obtained from trachea and oviduct, which express functional TRPV4 channels (7, 16, 17). Figure 2D shows typical oscillatory Ca2+ signals generated by hypotonic solutions in tracheal ciliated epithelial cells. However, Ca2+ response to hypotonicity was abrogated following the activation of PLC with 4α-PDD (Fig. 2F-G). The reduction in hypotonicity- and heat-induced Ca2+ signal were not due to Ca2+-dependent inhibition of TRPV4 as two consecutive stimuli elicited similar responses (Fig. S6A-B).

Similarly, mouse ciliated oviductal cells responses to hypotonicity were reduced following addition of ATP (Fig. S6C-D). Although we could not assess directly whether PIP2 remained depleted at the time cells were challenged with TRPV4 activating stimuli, the fact that there was no Ca2+ response to a second ATP stimulation within minutes of the first ATP application (Fig. S6E) may reflect a condition of PIP2 depletion.

Activity 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 cystolic messenger 5′-6′-epoxyeicosatrienoic 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 4α-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 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 TRPV4-121 AAFAA 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 PIP2, and consistent with previous reports (8, 21, 43), no significant change in channel activity was elicited by heat (Fig. 3E). Figure 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 Q10 obtained from excised patches containing TRPV4-WT in the presence of PIP2 was 21±5 (n=3) (Fig. S7B), 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 4α-PDD is not affected by PIP2 depletion or why PIP2 is unable to activate TRPV4 on its own.

PIP2 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 in these limited proteolysis assays on the purified TRPV4 N-terminal region (Fig. 4B-E) showed that proteolysis of TRPV4 N-terminus is limited proteolysis assays on the purified TRPV4 N-terminal region (Fig. 4B-E) showed that proteolysis of TRPV4 N-terminus is
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 interferes with the interaction of TRPV4 with PIP$_2$, an effect that was lost when a membrane-unbound PACSIN3-ΔF-BAR was used.

### 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 (in mM): 100 NaCl, 1 MgCl$_2$, 6 CaCl$_2$, 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, 4 NaATP and 0.1 NaGTP, 300 mOsm, pH 7.25. Bath and pipette solutions contained (in mM): 130 CsCl, 1 MgCl$_2$, 0.034 CaCl$_2$, 5 EGTA, 10 HEPES (310 mossmol/liter, pH 7.25). When required, solutions were warmed using a water jacket device (Warner Instruments). All chemicals were obtained from Sigma (St Louis, MO, USA) except CaCl$_2$ and MgCl$_2$ (Echelon Biosciences Inc.), HC-067047 (Tocris Biosciences) and Fura-2 (Invitrogen).

- 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/excision patches were perfused at 0.8 ml/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 were estimated as the increase of the FRET donor CF$^2$ 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, 1% glycerol, and 1 mM EDTA. Digestion was stopped at 15, 30, 45 and 60 min.

- Lipid protection assay by limited proteolysis

Patch-clamp whole-cell and single single-channel currents were recorded at room temperature (−25°C, unless otherwise indicated) as previously described (16, 48). Cells/excision patches were perfused at 0.8 ml/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).

Data are expressed as mean±SEM (or mean±S.D. in Fig. 4) of n experiments. Statistical analysis was assessed with Student’s unpaired t-test or one-way analysis of variance (ANOVA) using Sigma-Plot software.

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We thank Dr. T. Voets (KUL, Leuven, Belgium) for the gift of HEK-293 tetracycline-inducible phosphatase expressing cells, Dr. T. Meyer (Stanford, U.S.A.) for rat TRPV4 cDNA, Dr. M. Schaefer (Leipzig, Germany) for help with initial FRET experiments. This work was supported by the Spanish Ministry of Science and Innovation (SAF2012-38140), Fondo de Investigación Sanitaria (Red HERACLES RD12/0042/0014), FEDER Funds; Generalitat de Catalunya (SGR05-266); and National Institutes of Health (Grant R01 GM056148) to A.G-E. A.G.E. is the recipient of an ERC Starting Grant. A.G.E. is the recipient of a Ramón y Cajal Award. C.P., U.A.H. and R.V. performed research; A.G.E., S.M., C.P-P., H.I., U.A.H. and R.V. contributed equally to this work. 2 To whom correspondence should be addressed. E-mail: miguel.valverde@upf.edu

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A.G-E., A.E., G.V., J.V., and C.P-P. performed research; A.G-E., S.M., C.P-P., H.I., U.A.H. and R.V. contributed equally to this work. 2 To whom correspondence should be addressed. E-mail: miguel.valverde@upf.edu

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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 PIP2 biosensor (YFP-PE(PKC-δ)), the rapamycin-binding protein FRB and either the cytoplasmic enzyme construct FKBP-5-phosphatase (PI5-PEase) 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 PIP$_2$ 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 $^{121}$KRWRK$^{125}$ 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 phosphoinosides 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.