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 the cytosolic tails. Neutralization of the positive charges within the sequence 125KRWRK125, which resembles a phosphoinositide binding site, rendered the channel unresponsive to hypotonicity and heat but directly to osmotic stimuli, apart from the interaction between the TRPV4 activator andcalmodulin (23, 24), PACSIN3 (25), intracellular ATP (24) and epoxyeicosatrienoic acids (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 osmotic (20) and mechanical (7, 16) sensitivity of TRPV4 depends on the arachidonic acid metabolites, epoxyeicosatrienoic acids (EET), while the mechanism leading to temperature-mediated activation (only observed in intact cells) 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 and 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 atomic 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
screened TRPV4 for domains that may participate in the channel response to hypotonicity-induced cell swelling. A schematic view of the channel molecule and all deletions/mutations generated is shown in Supporting Information Fig. S1. We focused on the N-terminus tail because a TRPV4 SNP associated with hypotremia, P19S, generates a channel with reduced response to hypotonic cell swelling (37). We generated three deletions of different length: TRPV4-Δ1-30, TRPV4-Δ1-130 and TRPV4-Δ100-130.

TRPV4 sensitivity to hypotonic conditions was assayed using whole-cell patch clamp recordings and intracellular calcium imaging with fura-2. Addition of a 30% hypotonic solution yielded large currents in cells transiently transfected with TRPV4-WT but not in GFP-transfected cells (Fig. S2A). TRPV4-Δ1-30 and TRPV4-Δ100-130 responses to 30% hypotonicity were reduced to the levels recorded in GFP-transfected cells while TRPV4-Δ1-30 displayed a normal response (Fig. 1A and Fig. S2B). Deletion of the first 30 residues did greatly reduce currents generated by 15% hypotonic solutions (Fig. S2D), mimicking the changes induced by the P19S polymorphism (37). Consistent with the electrophysiological experiments, expression of TRPV4-Δ1-30 proteins increased intracellular [Ca^{2+}] in response to 30% hypotonicity while the response of TRPV4-Δ100-130 was indistinguishable from that obtained in GFP-transfected cells (Fig. S2C). To evaluate if differences in plasma membrane expression between WT and mutant TRPV4 proteins could explain the reduced responses of truncated TRPV4 proteins we determined surface labeling of HEK-293 cells expressing TRPV4-WT and TRPV4-Δ1-130 proteins tagged with a V5 epitope in the first extracellular loop. Confocal microscopy images and quantification by ELISA revealed no apparent differences in membrane expression between TRPV4-WT and the protein presenting the longest truncation, TRPV4-Δ1-130 (Fig. S3).

To pin down the region within residues 100-130 required for the channel response to hypotonic cell swelling we neutralized four positive charges within a sequence (KRWRK125) that has been proposed to be a possible phosphoinositide binding site (PI-site) (29). Cells transfected with TRPV4-Δ1-30 AAWAA displayed greatly reduced swelling-induced whole-cell cationic currents (Fig. 1B-C). TRPV4-Δ1-30 AAWAA generated currents in response to the synthetic agonist 4α-PDD (0.01-10 μM) were undistinguishable from TRPV4-WT currents (Fig. 1D). Sequential addition of a hypotonic solution and 10 μM 4α-PDD generated...
significant increases in intracellular Ca\textsuperscript{2+} levels in cells expressing TRPV4-WT while cells expressing TRPV4-\(\alpha\text{-PDD}\) did not reactivate TRPV4 (middle) while addition of 5,6-EET (10 µM) to TRPV4-WT responded to 4ºC imaging on cells exposed to a hypotonic solution (Fig. 3).

We hypothesized that the sequence \(\text{KRWRK}\) 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 \(\text{KRWRK}\) positive charges, we neutralized three positive charges of a nearby region (\(\text{121}^{\text{KRWRK}}\)). Expression of TRPV4 (\(\text{121}^{\text{AAA}}\)) produced a typical transient response to the levels obtained in GFP-transfected cells (Fig. 4A).

Depletion of PIP\textsubscript{2} levels prevents channel activation by psychophylic stimuli. We assessed whether deletion or mutation of residues \(\text{121}^{\text{KRWRK}}\) 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 cytosolic enzyme construct FKBP-S-phosphatase were co-transfected with TRPV4-WT in HeLa cells. Addition of
Activity of PIP$_2$-regulated channels typically decreases in excised inside-out patches and recovers 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. Hypotonicity-mediated activation of TRPV4 in excised patches can not be directly evaluated. Instead, the osmotransducing cytosolic messenger 5′,6′-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 4o-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 TRPV4-44 AAWAA 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$_{10}$ 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 4o-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 TRPV4-44 AAWAA 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 hypotonicity and heat but normal response to 4o-PDD (25, 44). 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 PI-site. Two compete hypotheses are that membrane-bound PACSIN3 binding to the PRD may either promote or physically block the interaction...
of the PI-site with membrane PIP₂. 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 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 CF₅ and YFP-tagged TRPV4 proteins, which we assumed formed a random population of heteromeric 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 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-Δ214AAWAA 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 PIP₂. To test this hypothesis, we studied how the reduction in PIP₂ levels affected FRET efficiency of TRPV4-WT. 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 enzyme depleted PIP₂ from the plasma membrane (Fig. S5D) and significantly increased FRET ratio (Fig. 5D). This observation further supported the hypothesis that mutations that prevented the N-terminal access to membrane PIP₂ (by deletion/mutation of the PI-site or by overexpression of PACSIN3) or depleted PIP₂ from the plasma membrane rearranged the cytosolic TRPV4 tails into a more compacted configuration (increased FRET ratio). Thus, in the presence of PIP₂, and an intact PI-site the intracellular tails appeared in an expanded conformation.

Conclusions. Together, our data provide several new findings. First, we have demonstrated that TRPV4, as many other TRP channels, is regulated by PIP₂, a process that involves PIP₂ binding to a PI-site (49) in the N-terminal. Second, we have showed that PIP₂ regulates channel activity in a stimulus-dependent manner. Third, TRPV4 is bona fide thermosensitive channel, providing there is PIP₂ to interact with the N-terminal. Fourth, the interaction of the TRPV4 PI-site with plasma membrane PIP₂ favors an expanded configuration of the intracellular tails and channel activation by hypotonicity and heat. Conditions that reduced PIP₂ levels (inducible phosphatase) or interfere the interaction of β-arrestins with PIP₂ (mutations in the PI-site or coexpression with PACSIN3) promote a compacted tail configuration and prevent channel activity by hypotonicity and heat. Our study provides 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 or oviductal ciliated 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 HEPEs, 0.1 NaATP, and 0.1 GTP, 300 mOsm, pH 7.25. Bath and pipette solutions for confocal imaging included: 130 GCl, 1 MgCl₂, 0.05 NaATP, 0.33 CaCl₂, 5 EGTA, 10 HEPEs (310 mosmol/liter, pH 7.25). When required, solutions were warmed using a water jacket device (Warner Instruments). All chemicals were obtained from Sigma-Aldrich except diC₈-PI and diC₈-PI(4,5)P₂ (Echelon Biosciences Inc.), HC-067047 (Tocris Biosciences) and Fura-2 (Invitrogen).

Electrophysiological and Ratiometric Ca²⁺ recordings

Patch-clamp whole-cell and single-channel currents were recorded at room temperature (−25°C, unless otherwise described) as previously described (16, 48). Cells/excision patches were perfused at 0.8 m/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 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-terminal (1-397) were cloned using Ndel and Noti into pET21-CGH (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-N-terminal, and 10 mM Tris-HCl pH 7.0, 150 mM NaCl and 1 mM DTT for TRPV4 ankyrin repeats. Lipid protection assay by limited proteolysis was performed at 4°C. (in ice) in reaction buffer containing (in mM): 180 NaCl, 20 Tris-HCl pH 7.0, 1% glycerol and 1 DTT (for TRPV4-1-397) or 150 NaCl, 20 Tris-HCl pH 7.0 and 1 DTT (for TRPV4-136-397 and TRPV4-ARYD). 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 μM, and 0.5 μg/ml respectively. Proteins were digested for 20 min 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±SD in Fig. 4) of n experiments. Statistical analysis was assessed with Student’s unpaired test or one-way analysis of variance (ANOVA) using Sigma-Plot software.


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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 chemiluminiscence 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-Phos) 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 PIP₃ biosensor (YFP-PH(PLC-δ)). The rapamycin-binding protein FRB and either the cytoplasmic enzyme construct FKBP-5-phosphatase (PIP₃-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 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 $^{121}\text{KRWRK}^{125}$ 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-$^{121}\text{AAWAA}$ 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.