IP$_3$ Receptor Binds to and Sensitizes TRPV4 Channel to Osmotic Stimuli via a Calmodulin-binding Site*

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Activation of the non-selective cation channel TRPV4 by mechanical and osmotic stimuli requires the involvement of phospholipase A$_2$ and the subsequent production of the arachidonic acid metabolites, epoxyeicosatrienoic acids (EET). Previous studies have shown that inositol trisphosphate (IP$_3$) sensitizes TRPV4 to mechanical, osmotic, and direct EET stimulation. We now search for the IP$_3$ receptor-binding site on TRPV4 and its relevance to IP$_3$-mediated sensitization. Three putative sites involved in protein-protein interactions were evaluated: a proline-rich domain (PRD), a calmodulin (CaM)-binding site, and the last four amino acids (DAPL) that show a PDZ-binding motif-like. TRPV4-PRD and CaM-CA128-381 channels preserved activation by hypotonicity, 4α-phorbol 12,13-didecanoate, and EET but lost their physical interaction with IP$_3$ receptor 3 and IP$_3$-mediated sensitization. Deletion of a PDZ-binding motif-like (TRPV4-ΔDAPL) did not affect channel activity or IP$_3$-mediated sensitization, whereas TRPV4-APRD (Δ132-144) resulted in loss of channel function despite correct trafficking. We conclude that IP$_3$-mediated sensitization requires IP$_3$ receptor binding to a TRPV4 C-terminal domain that overlaps with a previously described calmodulin-binding site.

The TRPV4$^2$ cation channel, a member of the TRP vanilloid subfamily, is expressed in a broad range of tissues where it participates in the generation of a Ca$^{2+}$ signal and/or depolarization of the membrane potential (1). TRPV4 participation in osmotic and mechanotransduction (1, 2) contributes to important functions including cellular (3, 4) and systemic volume homeostasis (5, 6), arterial dilation (7, 8), nociception (9), epithelial hyperelectrolyte transport (10, 11), bladder voiding (12), and ciliary beat frequency regulation (13–15). TRPV4 also responds to temperature (16, 17), endogenous arachidonic acid (AA) metabolites (18), and phorbol esters including the inactive 4α-phorbol 12,13-didecanoate (4αPDD) (19, 20) and participates in receptor-operated Ca$^{2+}$ entry (15), thus showing multiple modes of activation.

TRPV4 channel activity can be sensitized by co-application of different stimuli (9, 21, 22) or participation of different cell signaling pathways (14), suggesting the presence of different regulatory sites. In this sense, several proteins have been proposed to modulate TRPV4 subcellular localization and/or function: microtubule-associated protein 7 (23), calmodulin (CaM, 24), with no lysine protein kinase (25), and PACSIN3 (26). We have also recently reported a close functional and physical interaction between the inositol trisphosphate receptor 3 (IP$_3$R3) and TRPV4 that sensitizes the latter to the mechano- and osmotransducing messenger 5′-6′-epoxyeicosatrienoic acid (EET) (14).

This study, we reveal that IP$_3$-mediated sensitization of TRPV4 to EET requires IP$_3$R3 binding to TRPV4 and identifies the IP$_3$R3-binding site in a C-terminal region of the TRPV4 that coincides with a previously described CaM-binding site (24). Besides, our study has provided further evidence of the importance of the TRPV4 N terminus in channel gating as deletion of a proline-rich domain prevents channel activity by different stimuli despite correct localization and oligomerization.

**EXPERIMENTAL PROCEDURES**

*Generation of TRPV4 Mutants*—TRPV4 mutations were generated by site-directed mutagenesis (QuikChange® II XL site-directed mutagenesis kit, Stratagene) deleting: 13 amino acids (positions 132–144) to generate the mutation lacking the N-terminal PRD domain (TRPV4-APRD); 20 amino acids (positions 812–831) for the mutation lacking the calmodulin-binding domain (TRPV4-ΔCaM); and the last 4 amino acids of the channel (positions 868–871) for the mutation lacking the C-terminal PDZ domain (TRPV4-ΔDAPL). All TRPV4 constructs were tagged with YFP (or cyan fluorescent protein) at the C terminus, and sequences were verified by PCR.

*Cell Transfection*—HEK293 and HeLa cells were transiently transfected with ExGen500 (Fermentas MBI) using 8 μg of polyethylene enmine together with 3 μg of cDNA of the following plasmids: 1.5 μg of rat pcDNA3-IP$_3$R3 plasmid and 1.5 μg of human TRPV4-YFP plasmids, WT, or mutants. Cells were cultured from 24 to 48 h before use.

*Confocal Imaging*—HeLa cells transiently transfected with WT and TRPV4 mutants were fixed with 4% paraformaldehyde. The subcellular localization was analyzed under a ×63 1.32 oil Ph3 CS objective using an inverted Leica SP2 confocal microscope. Original images were not further processed except for adjustments of brightness, contrast, and color balance.

*Co-immunoprecipitation Experiments*—HEK293 cells were transiently transfected with IP$_3$R3 and TRPV4-YFP plasmids as described before. After 48 h, cells were lysed, total protein was

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2 The abbreviations used are: TRPV4, transient receptor potential vanilloid 4; AA, arachidonic acid, CaM, calmodulin, 4α-PDD, 4α-phorbol 12,13-didecanoate; EET, epoxyeicosatrienoic acid; IP$_3$, inositol trisphosphate; IP$_3$R, IP$_3$ receptor; PRD, proline-rich domain; YFP, yellow fluorescence protein; GFP, green fluorescent protein; WT, wild type; pf, picofarads.

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recollected, and Western blot assays were carried out (using 100 
µg) as described previously (14) to verify the presence of the 
proteins IP$_3$R3 and TRPV4 channel. Direct and reverse co-immuno-
precipitation experiments were done to evaluate the interaction etwenn both proteins. For this, 6 µg of anti-GFP (direct immuno-
precipitation) or anti-IP$_3$R3 (reverse immunoprecipitation) were 
incubated for 1 h with 30 µl of G protein (protein G-Sepharose 4B 
Fast Flow, Sigma-Aldrich) at 4 °C. 1500 µg of total protein samples 
from transfected and non-transfected cells were incubated over-
night at 4 °C, gently mixing, with the previous antibody-G protein 
complex. Immunocomplexes were washed four times in phos-
phate-buffered saline + 0.1% Nonidet P-40 solutions, and Western 
blot was performed. Primary antibodies used were anti-GFP 
(1:1000, mouse monoclonal antibody, Clontech Laboratories, Inc.) 
and anti-human TRPV4 (1:250, rabbit polyclonal antibody that 
recognizes the last 19 amino acids. Its specificity has been tested in 
both heterologous expression systems (27) and knock-out mouse 
models (12, 15)) for TRPV4-YFP and TRPV4 detection; and 
anti-IP$_3$R3 (1:2000, rabbit antibody, BD Biosciences) for IP$_3$R3 
detection.

**Calcium Imaging**—Intracellular calcium was determined in 
dtransfected HeLa cells loaded with 4.5 µM fura-2-AM (Molecular 
Probes, Leiden, The Netherlands) as described previously (3, 27). 
Cytosolic Ca$^{2+}$ levels are presented as the ratio of emitted fluores-
cence (510 nm) after excitation at 340 and 380 nm relative to the 
baseline. All experiments were performed at room temperature, 
and cells were bathed in an isotonic solution containing (in mM): 
140 NaCl, 5 KCl, 1.2 CaCl$_2$, 0.5 MgCl$_2$, 5 glucose, and 10 HEPES 
(∼305 mosmol/liter, pH 7.4).

**Electrophysiological Recordings**—Cationic currents were reg-
istered using the patch clamp technique in whole-cell configu-
ration. Patch pipettes were filled with a solution containing (in 
mM): 20 cesium chloride, 100 cesium acetate, 1 MgCl$_2$, 0.1 
EGTA, 10 HEPES, 4 Na$_2$ATP, and 0.1 NaGTP; 300 mosmol/liter, 
ph 7.2–7.3. Pipette solutions containing different concen-
trations of EET (BIOMOL) and IP$_3$ (Calbiochem) were also 
used and indicated in the corresponding figure (Fig. 4, B–F).

Cells were bathed in a solution containing (in mM): 140 NaCl, 5 
KCl, 1 MgCl$_2$, 10 HEPES, and 1 EGTA (∼310 mosmol/liter, pH 
7.3–7.4). HEK293 were clamped at 0 mV, and ramps from −140 
V mV to +100 mV (400 ms) were applied at a frequency of 0.2 Hz. 
Ramp data were acquired at 10 kHz and low-pass-filtered at 1 
kHz. All experiments were carried out at room temperature. 
Only those cells that presented YFP fluorescence were 
recorded.

**Statistics**—Data are expressed as the mean ± S.E. of n 
experiments. Analysis of variance or Student’s t test was per-
fomed with the Sigma Plot 8.02 (Systat Software, Inc.) and 
SPSS to determine statistical significance. Bonferroni’s test 
was used for post hoc comparison of means. p < 0.05 was 
considered significant.

**RESULTS AND DISCUSSION**

**Localization of Wild Type and TRPV4 Mutants**—We evaluated 
three putative IP$_3$R-interacting sites in TRPV4 that may 
participate in IP$_3$-mediated sensitization of TRPV4 to mechanical 
and osmotic stimuli (Fig. 1A). An N-terminal proline-rich domain 
(PRD), unique to TRPV4 and not present in other TRPV proteins, 
has been implicated in the channel interaction with the regulatory 
proteins PACSINs (28). A shorter C terminus PRD has also been 
identified in other members of the TRP family (29) and appears 
to participate in protein-protein interactions including the Homer 
family proteins and IP$_3$R, among others (30). TRPV4 also presents 
participate in protein-protein interactions including the Homer 
family proteins and IP$_3$R, among others (30). TRPV4 also presents 
participate in the interaction with IP$_3$R.

The subcellular distribution of wild type (TRPV4-WT, Fig. 1B) 
and TRPV4 mutants lacking the PRD (TRPV4-∆PRD) (Fig. 1C), 
the CaM (TRPV4-∆CaM) (Fig. 1D), or the final four amino acids 
(TRPV4-∆DAFL) (Fig. 1E) was examined by confocal microscopy 
former transiently transfected HeLa cells. Both WT and deletion 
TRPV4 mutants localized to the plasma membrane.

**Activation of WT and TRPV4 Mutants by Cell Swelling and 
4αPDD**—Intracellular [Ca$^{2+}$] measurements using the fura-2 dye 
indicator showed a clear response to 40% hypotonic solutions in 
transfected HeLa cells co-expressing TRPV4-WT, TRPV4-∆CaM, 
or TRPV4-∆DAFL constructs with IP$_3$R3 (Fig. 2A, C, and D). On restoration 
of the isotonic condition and recovering the basal [Ca$^{2+}$] levels, 
cells also triggered a robust Ca$^{2+}$ signal in response to 4αPDD (1 
µM), a synthetic activator of TRPV4 (19), thereby confirming the 
channels functional expression. However, cells overexpressing 
TRPV4-∆PRD did not respond to either 4αPDD (Fig. 2B) or hypo-
tonic solutions (Fig. 2B, inset) but showed a typical Ca^{2+} signal in response to the purinergic agonist ATP (100 μM).

Altogether, the results indicated that TRPV4-WT, TRPV4-ΔCam, and TRPV4-ΔDAPL used in this study trafficked to the plasma membrane where they responded to typical TRPV4-activating stimuli. On the other hand, TRPV4-ΔPRD, despite its correct localization, did not elicit Ca^{2+} signals in response to hypotonicity or 4μPDD and, therefore, was of no use for the identification of the molecular mechanism underlying IP_{3}-mediated sensitization of TRPV4. Nevertheless, TRPV4-ΔPRD revealed an additional piece of evidence suggesting the relevance of the N terminus in TRPV4 gating. A previous report has already pointed to the PRD as a functionally important domain for TRPV4 channel gating (26). D’Hoedt et al. (26) showed that combined mutation of P142A, P143A, and P152A resulted in channels unresponsive to CaM and ATP, respectively, and non-transfected (NT) cells (A, right panel) or non-transfected cells (B, left panel), TRPV4-ΔCam + IP_{3}R3 (A, center panel), or non-transfected (NT) cells (A, right panel) or non-transfected cells (B, left panel) had a typical Ca^{2+} signal by ATP was also observed in HEK293 cells expressing TRPV4-WT and IP_{3}R3 (center panel), or non-transfected cells (B, left panel), and immunocomplexes were analyzed by Western blotting with anti-IP_{3}R3 and anti-GFP. E-G, potentiation of TRPV4 response to hypotonic solutions by ATP. The top panels show representative intracellular Ca^{2+} signals (Δ ratio 340/380) obtained from cells transfected with the indicated constructs and exposed to the conditions shown in the bars. The bottom panels show mean increases (± S.E.) in 340/380 signal under the experimental conditions shown in the top panels. The conditions are: E (n = 33), F (n = 42), and G (n = 45), p < 0.05 between the presence or absence of ATP within groups was marked with * and p < 0.001 with **, paired t test.

The functional consequence of the loss of IP_{3}R binding to TRPV4-ΔCam on IP_{3}-mediated sensitization was first evaluated with the intracellular [Ca^{2+}] indicator fura-2. As reported previously (14), concomitant activation of the phospholipase A_{2}-AA-EET and phospholipase C-IP_{3} pathways using hypotonic solutions and ATP, respectively, resulted in TRPV4 sensitization. Fig. 3E shows Ca^{2+} signals obtained in HeLa cells expressing TRPV4-WT and IP_{3}R3 challenged with a short exposure to 30% hypotonic solution and then exposed to hypotonic solution containing 1 μM ATP (a concentration that does not trigger Ca^{2+} response in HeLa cells) (14). The addition of extracellular ATP potentiated the hypotonicity-induced Ca^{2+} response. Mean increases in the Ca^{2+} signal are shown in Fig. 3E (bottom). Potentiation of the hypotonicity-induced Ca^{2+} signal by ATP was also observed in HEK293 cells
co-expressing TRPV4-WT and IP$_3$R3 (not shown). Similar results were obtained examining the TRPV4-ΔDAPL construct that preserves IP$_3$R3 binding (Fig. 3G). However, TRPV4-ΔCaM channels retained their ability to respond to hypotonic solutions but showed no enhancement of the Ca$^{2+}$ signal when challenged a second time with hypotonic solutions containing ATP (Fig. 3F). In fact, similarly to TRPV4-WT-expressing cells exposed to a second hypotonic challenge in the absence of ATP (14), the second hypotonic shock (containing ATP) generated a reduced Ca$^{2+}$ signal in TRPV4-ΔCaM.

Deletion of the CaM-binding Site of TRPV4 Prevents IP$_3$-mediated Sensitization to EET—EET metabolites of arachidonic acid are the final activators of TRPV4 in response to hypotonic shocks (14, 36). Accordingly, TRPV4-expressing cells respond to either external (18) or internal EET-containing solutions (14), an effect that can be potentiated by IP$_3$ (14). In our attempt to characterize the importance of the CaM-binding site in the modulation of TRPV4 activation by osmotic stimulus, we studied the impact of deleting the CaM-binding site on the IP$_3$-mediated potentiation of EET-induced TRPV4 currents.

Whole-cell TRPV4 currents of equal magnitude were recorded from TRPV4-WT- and TRPV4-ΔCaM-expressing HEK293 cells exposed to 1 μM 4αPDD (Fig. 4A). Similarly, maximal TRPV4 currents recorded in HEK293 cells dialyzed with pipette solutions containing 156 nM EET were undistinguishable between TRPV4-WT- and TRPV4-ΔCaM-expressing cells (Fig. 4B). Mean responses measured at +100 mV in TRPV4-WT- and TRPV4-ΔCaM-expressing HEK293 dialyzed with 156 nM EET were 94 ± 22 pA/pF ($n=4$) and 79 ± 14 pA/pF ($n=7$), respectively ($p>0.05$). The electrophysiological data confirmed the observation carried out using intracellular Ca$^{2+}$ indicators that TRPV4-ΔCaM are functional channels responding to typical TRPV4 stimuli.

We have previously reported that low concentrations of EET (1 nM) or 30 μM IP$_3$ (added to the intracellular pipette solutions) hardly activate TRPV4 currents. However, the presence of both 1 nM EET and 30 μM IP$_3$ triggered sizeable TRPV4 currents in both native and heterologously expressing cell systems (14), indicative of the IP$_3$-mediated sensitization of TRPV4 to the mechanoo- and osmotransducing messenger EET. When 1 nM EET intracellular pipette solutions were tested on TRPV4-ΔCaM channels, no differences with TRPV4-ΔCaM channels were observed (26 ± 13 pA/pF ($n=5$) and 27 ± 12 pA/pF ($n=9$), respectively; measured at +100 mV; $p>0.05$) (Fig. 4C). A significant difference in current magnitude was detected between the response of TRPV4-WT and TRPV4-ΔCaM channels to the combined presence of 1 nM EET and 30 μM IP$_3$ in the intracellular pipette solutions (Fig. 4D). Considering the reported positive feedback of Ca$^{2+}$, via Ca$^{2+}$-CaM binding to TRPV4 (24), a possible alternative explanation for the absence of IP$_3$-mediated sensitization in TRPV4-ΔCaM channels may be the lost Ca-CaM binding. Although we considered this an unlikely possibility, as whole-cell patch clamp experiments were carried out in the absence of Ca$^{2+}$ and in the presence of EGTA (see also Ref. 14), this possibility was further ruled out using the CaM inhibitor W-7. The presence of the CaM inhibitor W-7 (30 μM) did not affect the IP$_3$-mediated sensitization of TRPV4-WT channels to 1 nM EET and 30 μM IP$_3$ (Fig. 4E). Mean responses measured at +100 mV in YFP-, TRPV4-WT-, and TRPV4-ΔCaM-expressing HEK293 dialyzed with 1 nM EET and 30 μM IP$_3$ are shown in Fig. 4F. Mean response of TRPV4-WT cells dialyzed with 1 nM EET, 30 μM IP$_3$, and 30 μM W-7 are also included.

**Conclusion**—Altogether, our results show that IP$_3$-mediated sensitization of TRPV4 is Ca-CaM independent, that TRPV4 channels lacking the CaM-binding site lose their interaction with IP$_3$R3, and consequently, they also lacked the IP$_3$-mediated sensitization to osmotic and direct EET stimulation. Our data with the TRPV4-ΔPRD also highlight the relevance of the prolinc-rich N-terminal region of TRPV4 in channel gating.

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