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# $IP_3$ 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, epoxieicosatrienoic 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-ΔCaM-(Δ812–831) 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-ΔPRD-(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 osmo- 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 hydroelectrolyte 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 kinases (25), and PACSIN3 (26). We have also recently reported a close functional and physical interaction between the inositol trisphosphate receptor 3 ($IP_3R3$) and TRPV4 that sensitizes the latter to the mechano- and osmotransducing messenger 5′-6′-epoxieicosatrienoic acid (EET) (14).

In this study, we reveal that $IP_3$-mediated sensitization of TRPV4 to EET requires $IP_3R3$ binding to TRPV4 and identify the $IP_3R3$-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 activation 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-ΔPRD); 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 eq of polyethyl enimine together with 3 μg of cDNA of the following plasmids: 1.5 μg of rat pcDNA3-$IP_3R3$ plasmid and 1.5 μg of human TRPV4-YPF 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 $IP_3R3$ and TRPV4-YPF 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; $IP_3R$, $IP_3$ 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 $\mu$g) as described previously (14) to verify the presence of the proteins $IP_3R3$ and TRPV4 channel. Direct and reverse co-immunoprecipitation experiments were done to evaluate the interaction between both proteins. For this, 6 $\mu$g of anti-GFP (direct immunoprecipitation) or anti-$IP_3R3$ (reverse immunoprecipitation) were incubated for 1 h with 30 $\mu$l of G protein (protein G-Sepharose 4B Fast Flow, Sigma-Aldrich) at 4 °C. 1500 $\mu$g of total protein samples from transfected and non-transfected cells were incubated overnight at 4 °C, gently mixing, with the previous antibody-G protein complex. Immunocomplexes were washed four times in phosphate-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-$IP_3R3$ (1:2000, rabbit antibody, BD Biosciences) for $IP_3R3$ detection.

*Calcium Imaging*—Intracellular calcium was determined in transfected HeLa cells loaded with 4.5 $\mu$M fura-2AM (Molecular Probes, Leiden, The Netherlands) as described previously (3, 27). Cytosolic $Ca^{2+}$ levels are presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm relative to the baseline. All experiments were registered 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 registered using the patch clamp technique in whole-cell configuration. 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 $Na_2ATP$, and 0.1 NaGTP; 300 mosmol/liter, pH 7.2–7.3. Pipette solutions containing different concentrations 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 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 performed 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 $IP_3R$-interacting sites in TRPV4 that may participate in $IP_3$-mediated sensitization of TRPV4 to mechanical and osmotic stimuli (Fig. 1*A*). An N-terminal proline-rich domain (PRD), unique to TRPV4 and not present in other TRPV proteins,

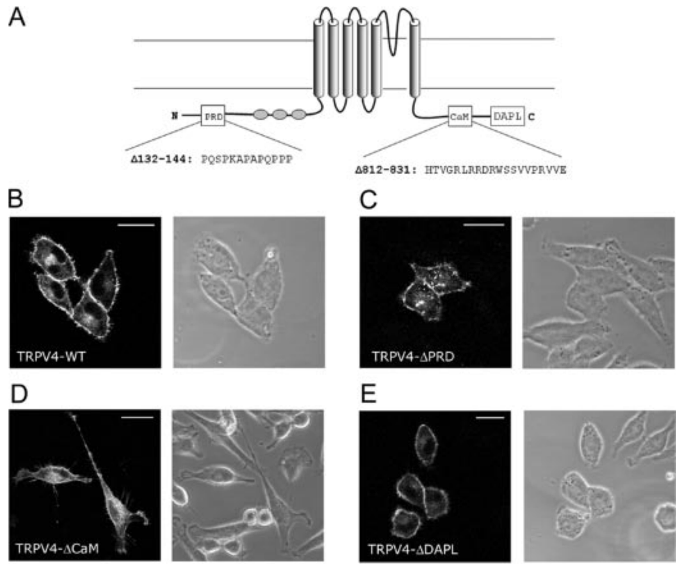


FIGURE 1. **Expression and localization of TRPV4 constructs.** *A*, schematic diagram of the TRPV4 protein showing the deletions introduced. *B–E*, immunofluorescence localization of TRPV4-WT (*B*), TRPV4-ΔPRD (*C*), TRPV4-ΔCaM (*D*), and TRPV4-ΔDAPL (*E*) in transiently transfected HeLa cells. Corresponding phase-contrast images are shown on the *right*. *Scale bar*, 10 $\mu$m.

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 $IP_3R$, among others (30). TRPV4 also presents a CaM-binding site on its C terminus (24). CaM-binding sites have been recognized in different TRP channels as important regulatory domains, exerting both inhibitory and facilitatory effects on TRPs upon $Ca^{2+}$-CaM binding (31). Besides, there is an overlap between the CaM- and $IP_3R$-binding sites in many TRPs (31), leading to the proposal that $IP_3R$ binding to and displacing CaM from its binding site contributes to the activation of canonical TRP (TRPC) channels (32). CaM binding to ankyrin repeats of TRPV1 has also been identified (33, 34), but we do not know at present whether this N-terminal CaM-binding site is also present in TRPV4. Finally, we checked whether the final four amino acids of TRPV4 (DAPL) that present a PDZ-binding motif-like (35), may participate in the interaction with $IP_3R$.

The subcellular distribution of wild type (TRPV4-WT, Fig. 1*B*) and TRPV4 mutants lacking the PRD (TRPV4-ΔPRD) (Fig. 1*C*), the CaM (TRPV4-ΔCaM) (Fig. 1*D*), or the final four amino acids (TRPV4-ΔDAPL) (Fig. 1*E*) was examined by confocal microscopy in 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 30% hypotonic solutions in HeLa cells co-expressing TRPV4-WT, TRPV4-ΔCaM, or TRPV4-ΔDAPL constructs with $IP_3R3$ (Fig. 2, *A*, *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 $\mu$M), a synthetic activator of TRPV4 (19), thereby confirming the channels functional expression. However, cells overexpressing TRPV4-ΔPRD did not respond to either 4αPDD (Fig. 2*B*) or hypo-

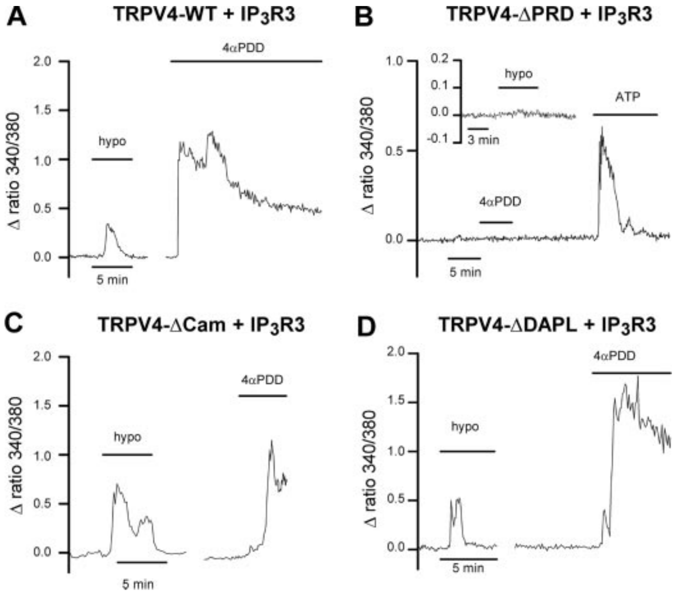


FIGURE 2. **Functional analysis of TRPV4 mutants.** Fura-2 ratios were obtained in HeLa cells co-transfected with TRPV4-WT + $IP_3R3$ (*A*), TRPV4-ΔCaM + $IP_3R3$ (*C*), and TRPV4-ΔDAPL + $IP_3R3$ (*D*) and sequentially stimulated with 30% hypotonic (*hypo*) solutions and 1 $\mu$M 4αPDD. The response of HeLa cells overexpressing TRPV4-ΔPRD + $IP_3R3$ (*B*) is shown separately for 1 $\mu$M 4αPDD and 100 $\mu$M ATP or 30% hypotonic solutions (*inset*). Traces are representative of $n > 100$ cells monitored from at least five independent experiments.

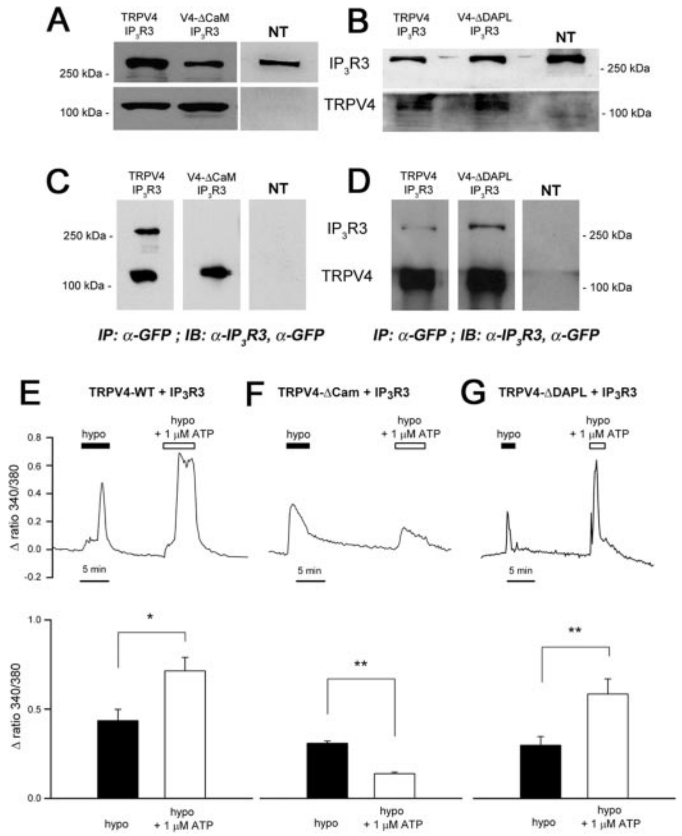


FIGURE 3. **Physical and functional interactions between TRPV4 constructs and $IP_3R3$.** *A* and *B*, detection of $IP_3R3$ (*top panels*) and TRPV4-YFP (*bottom panels*) in the cell extracts used for immunoprecipitation (*IP*) studies shown in *C* and *D*. Lanes were loaded with 100 $\mu$g of protein. *C* and *D*, TRPV4 was immunoprecipitated with anti-GFP monoclonal antibody from samples obtained from HEK293 cells transfected with TRPV4-WT + $IP_3R3$ (A, *left panel*), TRPV4-ΔCaM + $IP_3R3$ (*A*, *center panel*), or non-transfected (*NT*) cells (*A*, *right panel*) or TRPV4-WT + $IP_3R3$ (*B*, *left panel*), TRPV4-ΔDAPL + $IP_3R3$ (*B*, *center panel*), or non-transfected cells (*B*, *right panel*), and immunocomplexes were analyzed by Western blots (*IB*) with anti-$IP_3R3$ 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.

tonic solutions (Fig. 2*B*, *inset*) but showed a typical $Ca^{2+}$ signal in response to the purinergic agonist ATP (100 $\mu$M).

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 4αPDD 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 hypotonic shocks, AA, and heat that retained the response to 4αPDD. Our more drastic approach, deleting the entire PRD, in addition to preventing the response to hypotonicity also induced the loss of 4αPDD response.

*Deletion of the CaM-binding Site of TRPV4 Prevents $IP_3R$ Binding and Sensitization to Hypotonic Stimulus*—We have previously reported the modulatory effect of $IP_3R$ on TRPV4 activation by mechanical and osmotic stimuli as well as the immunoprecipitation of TRPV4 and $IP_3R3$ (not $IP_3R1$) (14). We now address the identification of the $IP_3R$-binding site on TRPV4 and its relevance for $IP_3$-mediated sensitization.

Expression of the proteins of interest in the lysates later used for co-immunoprecipitation studies was probed by Western blotting with anti-GFP (to detect TRPV4) and anti-$IP_3R3$ (Fig. 3, *A* and *B*). Co-immunoprecipitation experiments found that deletion of the CaM-binding site (V4-ΔCaM) abolished $IP_3R3$ binding (Fig. 3*C*), whereas deletion of the last four amino acids (V4-ΔDAPL) did not affect $IP_3R3$ binding (Fig. 3*D*). These results were further confirmed by reversed co-immunoprecipitation (results not shown).

The functional consequence of the loss of $IP_3R3$ 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. 3*E* shows $Ca^{2+}$ signals obtained in HeLa cells expressing TRPV4-WT and $IP_3R3$ challenged with a short exposure to 30% hypotonic solution and then exposed to hypotonic solution containing 1 $\mu$M 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. 3*E* (*bottom*). Potentiation of the hypotonicity-induced $Ca^{2+}$ signal by ATP was also observed in HEK293 cells co-expressing TRPV4-WT and $IP_3R3$ (not shown). Similar results were obtained examining the TRPV4-ΔDAPL construct that preserves $IP_3R3$ binding (Fig. 3*G*). 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. 3*F*). 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 $\mu$M 4$\alpha$PDD (Fig. 4*A*). 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. 4*B*). 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 $\mu$M $IP_3$ (added to the intracellular pipette solutions) hardly activate TRPV4 currents. However, the presence of both 1 nM EET and 30 $\mu$M $IP_3$ triggered sizeable TRPV4 currents in both native and heterologously expressing cell systems (14), indicative of the $IP_3$-mediated sensitization of TRPV4 to the mechano- and osmotransducing messenger EET. When 1 nM EET intracellular pipette solutions were tested on TRPV4-ΔCaM channels, no differences with TRPV4-WT 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. 4*C*). 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 $\mu$M $IP_3$ in the intracellular pipette solutions (Fig. 4*D*). 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 $\mu$M) did not affected the $IP_3$-mediated sensitization of TRPV4-WT channels to 1 nM EET and 30 $\mu$M $IP_3$ (Fig. 4*E*). Mean responses measured at +100 mV in YFP-, TRPV4-WT-, and TRPV4-ΔCaM-expressing HEK293 dialyzed with 1 nM EET and 30 $\mu$M $IP_3$ are shown in Fig. 4*F*. Mean response of TRPV4-WT cells dialyzed with 1 nM EET, 30 $\mu$M $IP_3$,and 30 $\mu$M W-7 are also included.

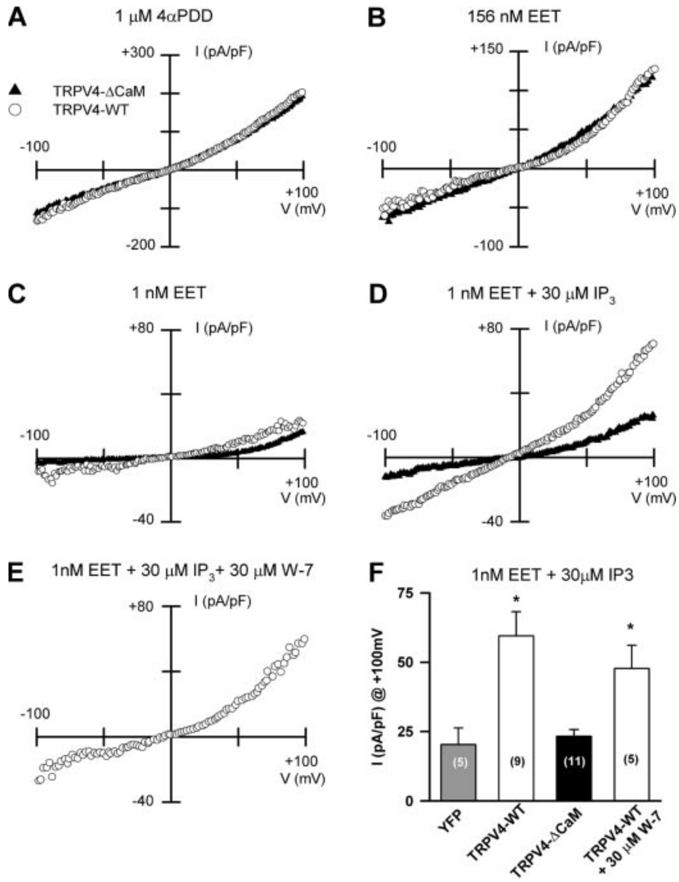


FIGURE 4. **TRPV4 CaM-binding site is required for $IP_3$-dependent sensitization to EET.** *A–E*, current-voltage relations of peak whole-cell cationic currents recorded from different HEK293 cells transfected with TRPV4-WT + $IP_3R3$ (○) or TRPV4-ΔCaM + $IP_3R3$ (▲) and exposed to 1 $\mu$M 4$\alpha$PDD (*A*), dialyzed with 156 nM EET (*B*), dialyzed with 1 nM EET (*C*), dialyzed with 1 nM EET + 30 $\mu$M $IP_3$ (*D*), or dialyzed with 1 nM EET + 30 $\mu$M $IP_3$ + 30 $\mu$M W-7 (*E*). *F*, mean current densities (±S.E.) at +100 mV in HEK293 cells expressing YFP ($n = 5$), TRPV4-WT + $IP_3R3$ ($n = 9$), and TRPV4-ΔCaM + $IP_3R3$ ($n = 11$) and dialyzed with 1 nM EET + 30 $\mu$M $IP_3$. Mean current density obtained from HEK cells overexpressing TRPV4-WT + $IP_3R3$ and dialyzed with 1 nM EET + 30 $\mu$M $IP_3$ + 30 $\mu$M W-7 ($n = 5$) are also shown. * $p < 0.05$ *versus* YFP, one way analysis of variance, and Bonferroni post hoc. No statistical difference was shown between TRPV4-WT and TRPV4-WT + W-7 ($p > 0.05$).

*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 $IP_3R3$, 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 proline-rich N-terminal region of TRPV4 in channel gating.

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