Stimulus-specific Modulation of the Cation Channel TRPV4 by PACSIN 3*

TRPV4, a member of the vanilloid subfamily of the transient receptor potential (TRP) channels, is activated by a variety of stimuli, including cell swelling, moderate heat, and chemical compounds such as synthetic 4α-phorbol esters. TRPV4 displays a widespread expression in various cells and tissues and has been implicated in diverse physiological processes, including osmotic homeostasis, thermo- and mechanosensation, vasorelaxation, tuning of neuronal excitability, and bladder voiding. The mechanisms that regulate TRPV4 in these different physiological settings are currently poorly understood. We have recently shown that the relative amount of TRPV4 in the plasma membrane is enhanced by interaction with the SH3 domain of PACSIN 3, a member of the PACSIN family of proteins involved in synaptic vesicular membrane trafficking and endocytosis. Here we demonstrate that PACSIN 3 strongly inhibits the basal activity of TRPV4 and its activation by cell swelling and heat, while leaving channel gating induced by synthetic ligands such as synthetic 4α-phorbol 12,13-didecanoate unaffected. A single proline mutation in the SH3 domain of PACSIN 3 abolishes its inhibitory effect on TRPV4, indicating that PACSIN 3 must bind to the channel to modulate its function. In line herewith, mutations at specific proline residues in the N terminus of TRPV4 abolish binding of PACSIN 3 and render the channel insensitive to PACSIN 3-induced inhibition. Taken together, these data suggest that PACSIN 3 acts as an auxiliary protein of TRPV4 channel that not only affects the channel's subcellular localization but also modulates its function in a stimulus-specific manner.

TRPV42 is a Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-permeable non-selective cation channel of the vanilloid-type transient receptor potential (TRP) channel subfamily (1–7). Like other TRP channels, it has six transmembrane-spanning (TM) domains with a putative pore region between TM5 and TM6, and cytoplasmic N and C termini (8, 9). TRPV4 is expressed in a broad range of tissues, including the lung, spleen, testis, fat, brain, cochlea, skin, smooth muscle, kidney, liver, and vascular endothelium (1, 2, 10–12). Due to its broad expression pattern and gating promiscuity, TRPV4 has the potential to play a role in diverse physiological processes (13). Indeed, using knockdown or knock-out strategies, a role for TRPV4 in osmotic homeostasis, thermosensing, pain, and vascular function has been firmly established (14–19). However, little is known about the mechanisms that regulate TRPV4 function in these different physiological settings, and it is unclear whether all known activation mechanisms are operating in every TRPV4-expressing cell. Heterologously expressed TRPV4 can be activated by a broad range of physical and chemical stimuli, including osmotic cell swelling (1, 2, 4, 20, 21), moderate heat (12, 22), synthetic ligands stimuli as 4α-phorbol 12,13-didecanoate (4α-PDD) (13, 23), mechanical force (14, 19, 24–26), fluid viscosity (27), and endogenous ligands such as anandamide and arachidonic acid (AA)-derived epoxyeicosatrienoic acids (28–30).

Several studies have provided evidence for regulation of TRPV4 expression and/or function by auxiliary proteins such as the AIP4 ubiquitin ligases, “With-No-K” kinase, PACSIN 3, and OS-9 (31–35). The PACSIN family consists of three members, PACSIN 1–3, which have been implicated in vesicle trafficking and endocytosis, although their exact biological relevance is unclear (36–39). TRPV4 binds most prominently to PACSIN 3, through an interaction between the C-terminal SH3 domain of PACSIN 3 and a proline-rich region in the N terminus of TRPV4, which leads to a reduction of the cytoplasmic concentration of TRPV4 resulting in an apparent relative increase of TRPV4's plasma membrane association (34).

In this study we reveal that PACSIN 3 not only affects the subcellular localization of TRPV4 but also modulates the sensitivity of TRPV4 to distinct stimuli. PACSIN 3 reduces the basal activity of TRPV4 and prevents activation by heat, cell swelling, and AA, whereas activation by 4α-PDD remains unaffected. Mutagenesis experiments further reveal that the modulation...
PACSIN 3 Modulates TRPV4 Activation

Effect of PACSIN 3 on TRPV1-expressing cells.

Measurement of \([Ca^{2+}]_i\) was performed using a monochromator-based imaging system consisting of a Polychrome IV monochromator (TILL Photonics, Martinsreid, Germany) and a Roper Scientific charge-coupled device camera connected to an Axiovert 200M inverted microscope (Zeiss, Germany). Monochromator and camera were controlled by Metafluor software (Version 6.3, Universal Imaging, Downingtown, PA). Fluorescence was measured during alternating excitation at 340 and 380 nm and corrected for the individual background fluorescence. The absolute \(Ca^{2+}\) concentration was obtained from the fluorescence ratios using the equation \([Ca^{2+}]_i = K_{eff}(R - R_0)/(R_1 - R)\), where \(K_{eff} = 2930\), \(R_0 = 0.18\), and \(R_1 = 6.0\) are calibration constants. \(R_0\) and \(R_1\) were estimated by perfusing cells with \(Ca^{2+}\)-free solution and high \(Ca^{2+}\) containing solution in the presence of \(1 \mu M\) ionomycin, respectively. The effective binding constant, \(K_{eff}\), was calculated by the equation, \(K_{eff} = K_0(R_1 + \alpha)/(R_0 + \alpha)\) with \(K_0\), the dissociation constant of Fura-2, and \(\alpha\), the isocoeficient. The \(K_d\) value was taken from Paltauf-Doburzynska and Graier (43). The isocoeficient \(\alpha\) was obtained as described by Zhou and Neher (44). The temperature of bath solutions was warmed by using a water jacket device (Warner Instruments); additionally a second external temperature sensor was used to control the bath solution.

Electrophysiology—Whole cell currents were measured with an EPC-10 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany, at a sampling rate, 1 ms; 8-Pole Bessel filter, 3 kHz) using ruptured patches. Patch electrodes had a DC resistance of 2–4 MΩ when filled with intracellular solution. An Ag-AgCl wire was used as reference electrode.

loration of TRPV4 activity requires binding of PACSIN 3 to a proline-rich region in the channel’s N terminus. Our results highlight the importance of the N terminus of TRPV4 in channel gating, and suggest that PACSIN 3 has a function comparable to that of the B-subunits of voltage-gated \(Ca^{2+}\) and \(Na^+\) channels (40–42).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum, 2 mM l-glutamine, 2 units/ml penicillin, and 2 mg/ml streptomycin at 37 °C in a humidity controlled incubator with 10% (v/v) CO₂. HEK-293 cells were transiently co-transfected with expression vectors encoding murine TRPV4 (Ensembl Gene ID: ENSMUSG00000014158) (30) or murine TRPV4 mutants and PACSIN 1 (ENSMUSG00000040276), PACSIN 2 (ENSMUSG00000016664), or PACSIN 3 (ENSMUSG00000027257) vectors in a 1:5 ratio. 12–18 h after transfections, the cells were seeded onto poly-l-lysine (Sigma)-coated coverslips and were further incubated for another 3 h before use.

FIGURE 1. PACSIN 3 inhibits heat-induced activation of TRPV4. A and B, intracellular calcium response to heat in cells transfected with TRPV4 (A) and TRPV4 co-expressed with PACSIN 3 (B). Gray lines and black lines represent non-transfected cell and transfected cells, respectively. C–F, time course at −80 mV (C) and 80 mV (D) in cells expressing TRPV4 (E) and TRPV4 with PACSIN 3 (F), application of 1 μM 4a-PDD after each experiment was used to confirm functional expression of the channel.

FIGURE 2. Effect of PACSIN 3 on TRPV1-expressing cells. A and B, \([Ca^{2+}]_i\), response to heat and capsaicin (100 nM) in cells expressing TRPV1 (A) and TRPV1 with PACSIN 3 (B). C, basal \([Ca^{2+}]_i\) level in non-transfected cells (n = 40), cells expressing TRPV1 (n = 36) and TRPV1 with PACSIN 3 (n = 32). D, average \([Ca^{2+}]_i\) increase in response to heat in non-transfected cells, TRPV1, and TRPV1 with PACSIN 3-transfected cells.
Capacitance and access resistance were monitored continuously. Between 50 and 70% of the series resistance (\( R_s \)) was electronically compensated to minimize voltage errors. A ramp protocol, consisting of a voltage step from a holding of 0 mV to +100 mV followed by a 400-ms linear ramp to +100 mV, was applied. This protocol was repeated every 5 s. Cell membrane capacitance (\( C_m = 6.0 \pm 0.3 \) picofarads (pF)) values were used to calculate current densities.

**Solutions**—For electrophysiological measurements, the standard extracellular solution contained (in mM): 150 NaCl, 6 CsCl, 1 MgCl\(_2\), 5 CaCl\(_2\), 10 glucose, 10 HEPES, buffered at pH 7.4 with NaOH. The Ca\(^{2+}\)-free pipette solution was composed of (in mM): 20 CsCl, 100 Cs-Asp, 1 MgCl\(_2\), 10 HEPES, 4 Na\(_2\)ATP, 10 BAPTA, buffered at pH 7.2 with CsOH. For measuring cell swelling-activated currents, we used a isotonic solution containing (in mM): 80 NaCl, 6 CsCl, 1.5 CaCl\(_2\), 1 MgCl\(_2\), 10 Hepes, 90 D-mannitol, 10 glucose, pH 7.4, resulting in 320 ± 10 mosmol. Cell swelling was induced by removing D-mannitol from the solution (giving 245 ± 10 mosM, a 25% reduction in osmolarity). The standard solution for calcium imaging experiments consisted of (in mM): 150 NaCl, 6 CsCl, 1 MgCl\(_2\), 1.5 CaCl\(_2\), 10 Hepes, 10 glucose, pH 7.4, buffered at a pH 7.4 with NaOH. The non-protein kinase C-activating phorbol ester, 4\(\alpha\)-phorbol 12,13-didecanate (4\(\alpha\)-PDD, Sigma) was applied at a 1 \(\mu\)M concentration from a 10 mM stock solution in ethanol. Arachidonic acid (Sigma) was used at a final concentration of 10 \(\mu\)M from a 10 mM stock solution in Me\(_2\)SO. Capsaicin was applied at a 100 nM concentration of a 1 mM stock solution in ethanol.

**Data Analysis**—Electrophysiological data were analyzed by using Patchmaster software (HEKA Elektronik, Lambrecht, Germany). Origin 6.1 software was used for statistical analyses and data display of electrophysiological and calcium imaging experiments. Data are expressed as mean ± S.E. Statistical analysis was performed with the Student’s \(t\) test.

**ROBETTA Modeling**—The N-terminal 470 amino acids of TRPV4 were used for fully automated prediction of the three-dimensional structure using the ROBETTA server (45, 46). The ROSETTA fragment insertion method was used to provide both \(ab\) \(initio\) and comparative models of protein domains (47). Comparative models were built from structures detected by PSI-BLAST or 3DJury-A1 and aligned by the K*Sync alignment method (48). The domain parsing and -fold detection were achieved using the Ginzu method (45, 49). Loop regions were assembled from fragments and optimized to fit the aligned template structure (50).

**FIGURE 3.** Effect of HTS and AA on \([Ca^{2+}]_i\) and whole cell currents in cells expressing TRPV4 and TRPV4 co-expressed with PACSIN 3. A and C, time course of \([Ca^{2+}]_i\) concentration in cells expressing TRPV4 alone after stimulation with HTS (A) or AA (C). B, I-V relation of whole cell currents at +80 mV ([]), and 80 mV (○) in TRPV4-expressing cells upon stimulation with HTS. D and F, intracellular calcium response in cells transfected with TRPV4 and PACSIN 3 to HTS (D) and AA (F). E, time course of the whole cell currents at +80 mV (□) and +80 mV (○) in cells co-expressing TRPV4 and PACSIN 3.

**FIGURE 4.** Effect of various stimuli on intracellular calcium in TRPV4 alone and TRPV4 co-expressed with PACSIN 3-expressing cells. A, basal \([Ca^{2+}]_i\) levels in non-transfected cells, and cells transfected with TRPV4 and TRPV4 with PACSIN3. B–E, average \([Ca^{2+}]_i\) increases induced by 4\(\alpha\)-PDD (B), HTS (C), AA (D), and heat (E) in non-transfected cells and in cells transfected with TRPV4, or TRPV4 with PACSIN 3 (\(n = 30–50\)). ***, \(p < 0.005\), significant difference compared with WT TRPV4-expressing cells.
RESULTS

PACSIN 3 Affects Heat-induced Activation of TRPV4—In a previous study it was shown that interaction with PACSIN 3 affects the subcellular distribution of TRPV4, which results in an apparent increase of the relative plasma membrane association of the channel (34). It remained untested whether PACSIN 3 affects channel gating. In this study, we show that PACSIN 3 co-immunoprecipitates endogenous TRPV4 from mouse kidney lysates, and we assess the interaction of various TRPV4 mutants with either PACSIN 2 or PACSIN 3 (supplemental Figs. S1 and S2). Finally, we used intracellular $\text{Ca}^{2+}$ ([Ca$^{2+}$]) measurements and whole cell patch clamp recordings to evaluate the effects of PACSIN 3 on basal TRPV4 activity and on the response to moderate heat, hypotonic cell swelling, and AA.

Transient expression of TRPV4 in HEK-293 cells results in spontaneous channel activity, which has been attributed to partial heat-activation at room temperature, and which leads to a significant but variable increase in basal [Ca$^{2+}$], (1, 2, 12, 30). Interestingly, co-expression of PACSIN 3 together with TRPV4 resulted in basal [Ca$^{2+}$] levels that were significantly lower than in cells expressing TRPV4 alone, and similar to the level observed in non-transfected HEK-293 cells, suggesting that PACSIN 3 inhibits spontaneous activity of TRPV4.

Heating the bath solution from 22 to 42 °C resulted in a robust increase of [Ca$^{2+}$], in TRPV4-expressing cells, which reverted to the baseline level when the temperature was brought back to 22 °C (Fig. 1A, for average [Ca$^{2+}$], values see Fig. 4), which is in agreement with previous studies (12, 22, 30). Subsequent application of 1 μM 4α-PDD to the same cells induced a second increase in [Ca$^{2+}$]. In contrast, co-transfection of PACSIN 3 fully abolished the [Ca$^{2+}$] response to heat, whereas the response to a subsequent 4α-PDD application remained unchanged (Fig. 1B, for average [Ca$^{2+}$], values see Fig. 4, B and E). Similar results were obtained in whole cell patch clamp experiments. In TRPV4-transfected cells, heating to 42 °C evoked typical TRPV4 currents (amplitude (n = 8): −47 ± 10 pA/pF (−80 mV) and 95 ± 6 pA/pF (80 mV); Fig. 1, C and E), whereas no heat-activated currents could be detected when PACSIN 3 was co-expressed with TRPV4 (amplitude (n = 6): −14.5 ± 4.5 pA/pF (−80 mV) and 25.1 ± 3.2 pA/pF (80 mV), p < 0.05, Fig. 1, D and F). TRPV1, TRPV2, and TRPV3 are closely related heat-activated channels, but lack the proline-rich domain required for the interaction of TRPV4 with PACSIN 3. In line therewith, we found that co-expression of PACSIN 3 had no effect on the sensitivity of TRPV1 to heat or capsaicin (100 nm) (Fig. 2). These results demonstrate that the effect of PACSIN 3 on heat-induced activation of TRPV4 is not a general property of heat-activated TRP channels.

Activation of TRPV4 by Cell Swelling Is Affected by PACSIN 3—Both in [Ca$^{2+}$], measurement and in whole cell recordings, application of a 25% hypotonic solution (HTS) evoked a robust response in TRPV4-expressing cells (Figs. 3A, 3B, and 4C). Strikingly, co-expression of PACSIN 3 completely abolished the response to HTS (Figs. 3D, 3E, and 4C) (after HTS stimulation: [Ca$^{2+}$], for TRPV4 (n = 20): 316 ± 20 nm, for TRPV4 plus PACSIN 3 (n = 30): 100 ± 19 nm, p < 0.005; amplitude for TRPV4 (n = 5): −52 ± 15 pA/pF (−80 mV) and 68 ± 14 pA/pF (80 mV), for TRPV4 plus PACSIN 3 (n = 5): −7 ± 2 pA/pF (−80 mV) and 13 ± 2 pA/pF (80 mV), p < 0.05). The mechanism for TRPV4 activation by cell swelling is distinct from heat- and 4α-PDD-induced channel activation. We have previously shown that swelling-induced activation of TRPV4 occurs via the PL$\alpha$- induced production of AA, which is further metabolized to epoxyeicosatrienoic acids that activate the channel in a membrane-delimited manner (28–30). Therefore, one possible explanation of the lack of HTS-induced TRPV4 activation could be a direct inhibitory effect of PACSIN 3 on the genera-
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FIGURE 6. Amino-terminal prolines in TRPV4 are involved in PACSIN 3 binding. A–C, [Ca\(^{2+}\)], response to heat (A), HTS (B), and AA (C) in cells expressing the TRPV4 double proline mutant, P142A-P143A. Cells still respond to all stimuli as well as to 4α-PDD. D–F, time course of Ca\(^{2+}\) concentration in cells co-expressing the TRPV4 mutant and PACSIN 3. Note, cells still respond to heat (G), HTS (H), and AA (I) in the presence of PACSIN 3. G–I, intracellular Ca\(^{2+}\) response to heat (G), HTS (H), and AA (I) in cells expressing the TRPV4 triple proline mutant, P142A/P143L/P152A. Transfected cells did not respond to heat, HTS, or AA but were still sensitive to 4α-PDD.

Previous studies have shown that [Ca\(^{2+}\)], has a strong modulatory effect on TRPV4 function and can cause both potentiation and inhibition of TRPV4 function (51, 52). This raised the question whether the strong inhibition of HTS-induced activation of TRPV4 in cells co-expressing PACSIN 3 would be related to the reduced basal [Ca\(^{2+}\)], in these cells. To investigate this possibility, we tested the effect of PACSIN 3 on TRPV4 mutant Y555A, which exhibits very low basal activity yet retains sensitivity to hypotonic cell swelling (30). Expression of TRPV4 mutant Y555A, resulted in a basal calcium level (109 ± 7 nM) similar to wild-type HEK-293 cells (103 ± 3 nM) or cells co-expressing the TRPV4 mutant Y555A, and PACSIN 3 (122 ± 7 nM) (supplemental Fig. S3). HTS stimulation of this mutant isoform resulted in an increase in [Ca\(^{2+}\)], (198 ± 12 nM). In contrast cells expressing both proteins, the TRPV4 mutant Y555A and PACSIN 3, did not respond any more to HTS stimulation (101 ± 5 nM) (supplemental Fig. S3). These results demonstrate that PACSIN 3 can inhibit HTS-induced activation of TRPV4 independent of its effect on basal channel activity.

A Single Proline Mutation in the SH3 Domain of PACSIN 3 Abolishes the Inhibitory Effect on TRPV4—PACSIN proteins have an SH3 domain at their C terminus, which interacts with proline-rich regions to mediate protein-protein interactions. Modregger and colleagues (39) demonstrated that mutation of a specific amino acid, Pro-415 to Leu [P415L], in the SH3 domain of PACSIN 3 abolished its inhibitory effect on endocytosis. Additionally, we previously showed that the same PACSIN 3 mutant lost its ability to interact with TRPV4 (34). To test the consequence of loss of this interaction, we co-transfected HEK-293 cells with TRPV4 and PACSIN 3 P415L and stimulated the cells with 4α-PDD, hypotonic solution, AA, or heat. Application of 4α-PDD caused an increase in [Ca\(^{2+}\)],. The basal and stimulated Ca\(^{2+}\) concentrations were 219 ± 18 nM and 424 ± 67 nM, respectively (Fig. 5, A, E, and F). Similarly, in whole cell patch clamp recordings we observed an increase in TRPV4 current in the presence of 4α-PDD. Current densities at −80 mV and +80 mV after the 4α-PDD stimulation were −280 ± 35 pA/pF and 480 ± 45 pA/pF, respectively (Fig. 5G). These current-density values were comparable with those obtained in cells that express TRPV4 alone (current densities after 4α-PDD stimulation: −335 ± 40 pA/pF (−80 mV) and 520 ± 60 pA/pF (+80 mV)). In addition, we tested whether cell swelling activates TRPV4 channels in the presence of the P415L PACSIN 3 mutant. Application of 25% hypotonic solution evoked an large increase in [Ca\(^{2+}\)], from 192 ± 19 nM to 429 ± 63 nM (Fig. 5B). In accord with these results, cell swelling also increased current size in cells that co-express TRPV4 and PACSIN 3 P415L (n = 5; from −5 ± 2 pA/pF (−80 mV) and 15 ± 3 pA/pF (+80 mV) before stimulation, to −40 ± 8 pA/pF and 71 ± 9 pA/pF at −80 mV and +80 mV after stimulation, respectively; Fig. 5H). These current-density values were comparable with those obtained with cells that expressed TRPV4 alone (n = 5; from −8 ± 1 pA/pF (−80 mV) and 15 ± 2 pA/pF (+80 mV) before stimulation, to −52 ± 15 pA/pF (−80 mV) and 68 ± 14 pA/pF after stimulation). Fig. 5D shows an AA-induced [Ca\(^{2+}\)], increase (380 ± 59 nM, n = 53) in cells that co-express TRPV4 and PACSIN 3 P415L. Similarly, in response to heat (between 22 °C and 42 °C) cells that express TRPV4 and PACSIN 3 P415L showed an increase in [Ca\(^{2+}\)], (92 ± 27 nM) (Fig. 5C), which was comparable with that of cells that express TRPV4 alone (133 ± 6276 JOURNAL OF BIOLOGICAL CHEMISTRY

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Notably, in our hands, basal \([\text{Ca}^{2+}]_i\) in cells that express TRPV4 and PACSIN 3 P415L was higher than that in cells that express TRPV4 and PACSIN 3, but comparable with that in cells that express TRPV4 alone (Fig. 5E). Taken together, these data suggest that co-expression of PACSIN 3 P415L with TRPV4 does not influence the basal activity of the channel and the activation of TRPV4 by several different stimuli.

The N-terminal Proline-rich Domain of TRPV4 Is Important for the PACSIN 3-mediated Effect—A proline-rich region in the N-terminal part of TRPV4 participates in interaction with the SH3 domain of PACSIN 3 (see Fig. 9A), and the combined mutation of prolines at positions 142 and 143 to alanine and leucine, respectively (mutant isoform P142A/P143L), has previously been shown to abolish the interaction with PACSIN 3 (34). In \([\text{Ca}^{2+}]_i\) imaging experiments, the P142A/P143L mutant isoform responded to stimulation with 4\(\alpha\)-PDD, heat, HTS, or AA independent of the presence of PACSIN 3 (Figs. 6(A–F) and 8(B and C) and Table 1). Moreover, cells expressing the P142A/P143L mutant displayed elevated basal \([\text{Ca}^{2+}]_i\) levels, both in the absence and presence of PACSIN 3, indicating that PACSIN 3 has no effect on the basal activity of this mutant (see Fig. 8A). Similar results were obtained with single mutations at these positions (mutants P142A and P143L). We found that these two mutants no longer bind PACSIN 3, and concomitantly that PACSIN 3 had no inhibitory effect on their activation (Figs. 7(A, B, D, E, G, H, J, and K) and 8(E and F) and Table 1). As a control for the specificity of the effects of the TRPV4 proline mutations, we mutated several neighboring prolines that are not required for the interaction with PACSIN 3. Single TRPV4 mutant, P152A, as well as...
the combined P132A/P135L mutant displayed normal responses to all tested stimuli, whereas co-expression of PACSIN 3 reduced their basal activity and caused a selective loss of sensitivity to HTS and AA stimulation (Figs. 7 (C, F, I, and L) and 8 (B, C, E, and F) and Table 1).

Surprisingly, combined mutation of three prolines in the proline-rich region of TRPV4 (mutant P142A/P143L/P152A) led to a channel exhibiting low basal activity and a complete loss of sensitivity to heat, HTS, and AA, independent of the expression of PACSIN 3. The response of this mutant to 4α-PDD, however, remained unaffected, indicating that its plasma membrane expression is not affected (Figs. 6 (G–I) and 8 (A–C) and Table 1). Possibly, simultaneous mutation of the three prolines leads to misfolding of the proline-rich N-terminal region, thus abolishing the channel's ability to respond to cell swelling and heat. Even without knowledge of the precise structural consequences of these mutations, the results highlight the functional importance of the N terminus of TRPV4.

**DISCUSSION**

Our experiments identified a novel role for PACSIN 3 as a stimulus-dependent modulator of the cation channel TRPV4. [Ca²⁺], measurements and patch clamp recordings demonstrated that PACSIN 3 inhibits the basal activity of TRPV4 as well as its responses to heat and HTS, whereas activation by 4α-PDD remained unaffected. In addition, detailed mutagenesis revealed that the modulatory action of PACSIN 3 requires a direct interaction between its SH3 domain and a unique proline-rich domain in the N terminus of TRPV4.

Previous work had already indicated that HTS, heat, and 4α-PDD use distinct mechanisms to activate TRPV4 (30). In particular, evidence was presented that transmembrane segments 3 and 4 (S3–S4) are involved in the activation of the channel by 4α-PDD and related α-phor-
bols, suggesting that this region forms an interaction site for these ligands (13, 30). Importantly, mutants in the S3–S4 region that affected 4α-PDD sensitivity were mostly without effect on channel activation by HTS. Instead, HTS-induced activation of TRPV4 was found to depend on the phospholipase A2-dependent release of AA, and the subsequent cytophrome P450-dependent conversion of AA to epoxyeicosatrienoic acids (28–30). Two lines of evidence indicate that the N terminus of TRPV4 is an important determinant of HTS-induced AA-dependent channel activation. First, binding of PACSIN 3 to this region completely suppresses this mode of channel activation, and second, a triple mutation in this region (P142A/P143L/P152A) renders the channel insensitive to HTS and AA (but not to 4α-PDD), independently of the presence of PACSIN 3. It is interesting that the response of TRPV4 to heat is not only sensitive to mutations that affect 4α-PDD sensitivity (13, 30) but is also inhibited by the binding of PACSIN 3, suggesting that the thermal sensitivity of TRPV4 depends both on the S3–S4 region and the N terminus.

The proline-rich N-terminal region of TRPV4, which is important for the interaction with PACSIN 3, does not have a counterpart in other members of the TRPV subfamily (Fig. 9A). Consequently, PACSIN 3 does not interact with TRPV1 or TRPV2 (34), and we found that co-expression of PACSIN 3 does not affect the sensitivity of TRPV1 to heat or capsaicin. It would be of great interest to understand the structure of TRPV4 N terminus and its unique proline-rich PACSIN 3-interaction site, but currently the quantity of structural data for TRP channels is limited. As a first approach, we have performed a homology modeling and an ab initio structure determination of the N-terminal amino acid sequence of TRPV4 (Fig. 9B). The resulting tentative model shows an L-shaped structure with a curve just preceding the first putative ankyrin motif. The first 200 amino acids of TRPV4 seem to be packed in rather compact globular structure, whereas the second part displays a stem-like arrangement of ankyrin repeats similar to the crystal structure of the corresponding region in TRPV2 (53, 54). The proline-rich region involved in the interaction with PACSIN 3 is located close to the bending point between the extreme N terminus and the ankyrin domain. It is tempting to speculate that HTS- or heat-induced gating of TRPV4 may involve a hinge movement in this region, and that binding of PACSIN 3 interferes with this movement. In this context, the unresponsiveness to HTS or heat of the triple mutation P142A/P143L/P152A could be due to a misfolding of this region. Clearly, additional functional and crystallographic data will be required to understand how structural rearrangements of the TRPV4 N terminus affect channel gating.

The effects of the cytosolic PACSIN 3 on TRPV4 are reminiscent of those of the β-subunits of voltage-gated CaV$^{2+}$, Na$^{+}$, and K$^{+}$ channels. For example, β-subunits (Ca$_{v}$β) of voltage-gated Ca$^{2+}$ channels are SH3 domain-containing cytosolic proteins that interact with an intracellular loop of the pore-forming α-subunits (Ca$_{v}$α) (42). This interaction not only affects the membrane expression of the α-subunits, but also profoundly alters their gating properties, including the voltage dependence of channel activation and inactivation, as well as its modulation by G proteins and pharmacological agents. Levels of Ca$_{v}$β sub-units are dynamically and developmentally regulated and contribute greatly to the functional variability of voltage-gated CaV$^{2+}$ channels in different cells and tissues (41). Further experiments should be devoted to examine whether PACSIN 3 has a similar modulatory action on TRPV4 in vivo.

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