Determinants of 4α-Phorbol Sensitivity in Transmembrane Domains 3 and 4 of the Cation Channel TRPV4

Joris Vriens, Grzegorz Owsiianik, Annelies Janssens, Thomas Voets, and Bernd Nilius

From the Department of Physiology, Campus Gasthuisberg, KU Leuven, B-3000 Leuven, Belgium

TRPV4, a Ca2+-permeable member of the vanilloid subgroup of the transient receptor potential (TRP) channels, is activated by cell swelling and moderate heat (≥ 27 °C) as well as by diverse chemical compounds including synthetic 4α-phorbol esters, the plant extract bisandrographolide A, and endogenous epoxyeicosatrienoic acids (EETs; 5,6-EET and 8,9-EET). Previous work identified a tyrosine residue located in the first half of putative transmembrane segment 3 (TM3) as a crucial determinant for the activation of TRPV4 by its most specific agonist 4α-phorbol 12,13-didecanoate (4α-PDD), suggesting that 4α-PDD interacts with the channel through its transmembrane segments. To obtain insight in the 4α-PDD-binding site and in the mechanism of ligand-dependent TRPV4 activation, we investigated the consequences of specific point mutations in TM4 on the sensitivity of the channel to different chemical and physical stimuli. Mutations of two hydrophobic residues in the central part of TM4 (Leu584 and Trp590) caused a severe reduction of the sensitivity of the channel to 4α-PDD, bisandrographolide A, and heat, whereas responses to cell swelling, arachidonic acid, and 5,6-EET remained unaffected. In contrast, mutations of two residues in the C-terminal part of TM4 (Tyr591 and Arg594) affected channel activation of TRPV4 by all stimuli, suggesting an involvement in channel gating rather than in interaction with agonists. Based on a comparison of the responses of WT and mutant TRPV4 to 4α-PDD and different 4α-phorbol esters, we conclude that the length of the fatty acid moiety determines the ligand binding affinity and propose a model for the interaction between 4α-phorbol esters and the TM3/4 region of TRPV4.

TRPV4 is a widely expressed cation channel of the “transient receptor potential” (TRP) superfamily (1–6). TRP channels are intrinsic membrane proteins with six putative transmembrane spans (TM) and a cation-permeable pore region formed by a short hydrophobic stretch between TM5 and TM6. TRPV4 functions as a Ca2+-entry channel that exhibits a surprising gating promiscuity. The channel can be activated by physical stimuli (cell swelling and moderate warmth (≥ 27 °C) (3, 8–13)), by the synthetic phorbol ester 4α-PDD (14), by the active compound of an extract from the plant Andrographis paniculata, bisandrographolide A (BAA) (15), and by EETs derived from arachidonic acid (AA) (3, 16). Cell swelling activates TRPV4 by means of the phospholipase A2-dependent formation of AA, which is subsequently metabolized to various EETs via cytochrome P450 epoxygenases (2). Phorbol esters and heat operate via a distinct, phospholipase A2- and cytochrome P450 epoxygenase-independent pathway, which critically depends on an aromatic residue at the N terminus of TM3, Tyr556 (referred to as Tyr555 in a previous publication) (17). In mouse aorta endothelial cells, modulation of CYP2C expression enhanced the responses to AA and cell swelling, whereas responses to other stimuli remained unaffected (18). Relatively few reports describe the functional impact of TM1–4 segments on the regulation of the TRP channel function (6). The first insights came from a study on TRPV1 (19), suggesting that Tyr511 in the intracellular loop between TM2 and TM3 interacts with the vanillyl moiety of capsaicin on the cytosolic face of the membrane. Nearby residues, such as Ser512 or Arg591, may interact with capsaicin via hydrogen bonds, whereas lipophilic residues in TM3 can be involved in stabilization via hydrophobic interactions with the aliphatic moiety of capsaicin within the plane of the membrane (19). This model was refined by Gavva et al. (20), who demonstrated that Tyr511, Trp550, and Met547 in TM4 might be involved in the interaction with the vanilloid moiety, whereas the aliphatic tail of capsaicin binds to Tyr511. Nevertheless, these two models do not explain the loss of capsaicin sensitivity by mutations of N- and C-terminal residues, Arg114 and Asp761, in TRPV1 (21, 22) and still require additional biochemical and structural data for validation.

In the present study, we identify residues in TM4 of TRPV4 that are critically involved in ligand binding. Moreover, by comparing the effects of 4α-phorbol with different aliphatic side chains, we obtained the first insights into the orientation of these ligands and their interaction with TM3/TM4 region of TRPV4.

12,13-didecanoate; 4α-PDD, 4α-phorbol 12,13-dibutyrate; 4α-PMA, 4α-phorbol 12-myristate 13-acetate; 4α-PDA, 4α-12,13-diacetate; BAA, bisandrographolide A; AA, arachidonic acid; HEK, human embryonic kidney; GFP, green fluorescence protein; WT, wild type.

VOLUME 282 • NUMBER 17 • APRIL 27, 2007

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 17, pp. 12796 –12803, April 27, 2007 © 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney cells, HEK293, were grown in Dulbecco’s modified Eagle’s medium containing 10% (v/v) human serum, 2 mM L-glutamine, 2 units/ml penicillin, and 2 mg/ml streptomycin at 37 °C in a humidity-controlled incubator with 10% CO2. HEK293 cells were transiently transfected with the murine TRPV4 (mouse mTRP12; accession number Q9EPK8) bicistronic pCAGGS/IREGFP vector (12) using Mirus TransIT-293 (Mirus Corp.; Madison, WI). Transfected cells were visualized by green fluorescence protein (GFP) expression, whereas GFP negative cells from the same batch were used as controls.

Site-directed Mutagenesis—All mutants were obtained by the standard PCR overlap extension method (23) using mTRPV4 from pCAGGS/IREGFP vector. Accuracy of all mutant sequences was verified by sequencing. Expressions of mutant channels were verified by cell surface biotinylation and immunodetection with anti-TRPV4 antibodies (see supplemental Fig. 1).

Solutions—For electrophysiological measurements, the standard extracellular solution contained (in mM): 150 NaCl, 6 CsCl, 1 MgCl2, 5 CaCl2, 10 glucose, 10 HEPS, buffered at pH 7.4 with NaOH. The osmolarity of this solution, as measured with a vapor pressure osmmeter (5 Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mosm. When measuring swelling-activated currents, we used an isotonic solution containing (in mM): 105 NaCl, 6 CsCl, 5 CaCl2, 1 MgCl2, 10 HEPS, 90 d-mannitol, 10 glucose, buffered pH 7.4 with NaOH (320 ± 5 mosm). Cell swelling was induced by omitting mannitol from this solution (240 mosm, 25% reduction of osmolarity). The pipette solution was composed of (in mM): 20 CsCl, 10 Asp, 1 MgCl2, 10 HEPS, 4 Na2ATP, 10 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetate (BAPTA), and 0.08 CaCl2 buffered at pH 7.2 with CsOH. The free Ca2+ concentration of this solution is ~200 nM. The non-PKC-activating phorbol ester 4α-PDD (Sigma) was applied at the indicated concentrations from a 10 mM stock solution in ethanol. Other 4α-phorbol derivatives, such as 4α-phorbol 12,13-dibutyrate (4α-PDBu; PKC Pharmaceuticals) and 4α-phorbol 12-myristate 13-acetate (4α-PMA, Sigma), were applied at the indicated concentrations from a 50 mM stock solution in ethanol. 12,13-Diacetate (4α-PDA; MPBiomedicals) and 4α-phorbol (PKC Pharmaceuticals) were applied from a 100 mM stock solution in ethanol. AA (Sigma) was used at a concentration of 10 μM from a 10 mM stock solution in water, and 5,6-EET (Biomol) was applied to the bath solution at a concentration of 500 nM. In the case of activation by heat, the perfusate was warmed using a water jacket device. We changed the temperature in the bath from 22 to 43 °C in 50 s. Other experiments were performed at room temperature (22–25 °C).

Electrophysiological Recordings—Whole-cell membrane currents were measured with an EPC-10 (HEKA Elektronik, Lambrecht, Germany) using ruptured patches with samples rate of 20 kHz, and currents were filtered at 2.9 kHz. Patch electrodes had a DC resistance between 2 and 4 megohms when filled with intracellular solution. An Ag–AgCl wire was used as a reference electrode. Capacitance and access resistance were monitored continuously. Between 50 and 70% of the series resistance was electronically compensated to minimize voltage errors. We applied a ramp protocol consisting of a voltage step from the holding potential of 0 mV to −100 mV followed by a 400-ms linear ramp to +100 mV. This protocol was repeated every 2 s. Step protocol consists of 20-mV steps from −100 mV to +100 mV. Mean cell membrane capacitance value was 7.65 ± 0.1 picofarads for n = 78 and very similar for all HEK cells; therefore, current densities are not calculated.

Measurement of Intracellular Ca2+ Concentrations—Intracellular [Ca2+]i was measured with a monochromator-based imaging system described in detail elsewhere (24). For every condition, at least 20 cells from at least three independent experiments were assayed. The calibration procedure was described previously in (24). For experiments with responses to heat, Keff for changes in temperature was corrected (25).

Data Analysis—Electrophysiological data were analyzed using the PATCHMASTER and FITMASTER programs (HEKA Elektronik). For statistical analysis and data display, the Origin 7.1 software package was used (OriginLab Corp., Northampton, MA). Dose-response relationships were fitted with a Hill equation of the form,

\[
\Delta\text{Ca}^{2+} = \frac{\Delta\text{Ca}_{\text{max}}^{2+}}{1 + \left(\frac{\text{EC}_{50}}{C}\right)^{n_H}} \quad \text{(Eq. 1)}
\]

where C represents the agonist concentration, EC50 represents the concentration for half-maximal response, and nH represents the Hill coefficient. Data are expressed as mean ± S.E., and significance (p < 0.01) between individual groups was tested using unpaired Student’s t test.

RESULTS

Sequence alignment of the putative TM3 and TM4 of TRPV4 (residues 547–570) with the corresponding region of TRPV1 displayed a high level of similarity (60% homology/40% identity) (Fig. 1). Importantly, Leu584, Trp586, and Met587 in TM4 of TRPV4 aligned with residues in TRPV1 that were previously described to contribute to vanilloid binding (20). Moreover, Asn588, Tyr591, and Arg594 were also highly conserved in other TRPV members. To assess contribution of these residues to TRPV4 activation, we constructed a series of point mutations from pCAGGS/IRES-GFP vector. Accuracy of all mutant sequences was verified by sequencing. Expressions of mutant channels were verified by cell surface biotinylation and immunodetection with anti-TRPV4 antibodies (see supplemental Fig. 1).

Mutations to the Central Part of TM4 Affect Responses to Phorbol Esters and Heat—When compared with wild type TRPV4, mutants L584M and W586A displayed a high level of similarity (60% homology/40% identity) (Fig. 1). Importantly, Leu584, Trp586, and Met587 in TM4 of TRPV4 aligned with residues in TRPV1 that were previously described to contribute to vanilloid binding (20). Moreover, Asn588, Tyr591, and Arg594 were also highly conserved in other TRPV members. To assess contribution of these residues to TRPV4 activation, we constructed a series of point mutations within TM4 and tested the response of the mutant channel expressed HEK293 cells to the different known TRPV4 stimuli using intracellular Ca2+ ([Ca2+]i) measurements and whole-cell patch clamp recordings.

Mutations to the Central Part of TM4 Affect Responses to Phorbol Esters and Heat—When compared with wild type TRPV4, mutants L584M and W586A displayed significantly reduced [Ca2+]i increases and inward TRPV4 currents in response to 5 and 10 μM 4α-PDD (Fig. 2, A, B, and D), together with an ~10-fold increase in EC50 (Table 1). In contrast, single mutations to the nearby residues Met587 and Asn588 (mutants M587A and N588A) mutants had no obvious effect on the [Ca2+]i responses to 4α-PDD (Fig. 2A and Table 1). However, the combined mutant W586A,M587A exhibited a higher EC50 value than the W586A single mutant (Fig. 2C and Table 1).
Tyrosine 556 in TM3 was previously identified as a critical residue for activation of TRPV4 by 4µM-PDD and heat, based on the finding that substituting a nonaromatic amino acid at this position abolished the response to 1 µM 4µM-PDD and heating to 42 °C (17). However, we found that 4µM-PDD concentration of 5µM and higher evoked significant [Ca²⁺], responses and whole-cell currents in cells expressing Y556A and determined an EC₅₀ of 6.3 µM (Fig. 2, A and B, and Table 1). Interestingly, combining mutation Y556A with mutations in TM4 led to a further decrease (mutant Y556A,M587A) or even complete loss (mutant Y556A,W586A) of 4α-PDD sensitivity (Fig. 2E and Table 1). Taken together, these results indicate that residues in both TM3 and TM4 cooperate in the 4α-PDD-dependent stimulation of TRPV4.

BAA, derived from the plant A. paniculata and used in traditional Chinese medicine as an anti-inflammatory, immunostimulant, and antihypertensive compound, was recently described as a new agonist of TRPV4 that activates the channel in a membrane-delimited manner (15). We tested whether the above described mutants with reduced 4α-PDD sensitivity were also affected in their responses to BAA. We found that mutants L584M and W586A were fully unresponsive to BAA over a broad range of concentrations (Fig. 3, B and C). In contrast, the response of Y556A to BAA was indistinguishable from that of WT TRPV4, with an EC₅₀ of about 1.2 ± 0.2 µM (Fig. 3C).

In the absence of a clear stimulus, TRPV4 displays some basal activity that can be estimated from the spontaneous single-channel openings in cell-attached patches (8), the increased basal whole-cell currents shortly after establishment of the whole-cell configuration (12, 26), and the higher basal Ca²⁺ levels in TRPV4 transfected cells (8, 16, 17). We found that basal [Ca²⁺], levels in HEK cells expressing the mutants L584M, W586A, Y556A, Y556A,W586A, W586A,M587A, and Y556A,M587A were similar to that of nontransfected cells, which contrasts to the elevated basal [Ca²⁺], levels in TRPV4-transfected cells (Fig. 4A). Moreover, none of these mutants showed a [Ca²⁺], response to a heat stimulus of 42 °C (Fig. 4B).

Two lines of evidence indicate that the strong impairment of the responses to 4α-PDD and heat upon mutating Leu 584,
TABLE 1
Comparison of agonist activation of TRPV4 and mutants

| Mutant      | \(\Delta [Ca^{2+}]_i\) | \(\Delta I_{-80\, mV}\)|
|-------------|------------------------|---------------------|
| NT          | No response            | No response         |
| WT TRPV4    | 0.72 ± 0.2 \(\mu\)m     | <1 \(\mu\)m         |
| Y556A       | 6.3 ± 0.3 \(\mu\)m     | 7.5 ± 1 \(\mu\)m     |
| W586A       | >6 \(\mu\)m             | 16 ± 2 \(\mu\)m      |
| Y556A,W586A | No response             | No response         |
| M587A       | <1 \(\mu\)m             | <1 \(\mu\)m         |
| W586A,M587A | >10 \(\mu\)m            | 32 ± 10 \(\mu\)m    |
| Y556A,M587A | >6 \(\mu\)m             | 17 ± 9 \(\mu\)m      |
| N588A       | <1 \(\mu\)m             | <1 \(\mu\)m         |
| Y591A       | >25 \(\mu\)m            | 30 ± 2 \(\mu\)m      |
| R594A       | No response             | No response         |
| R594Q       | No response             | No response         |
| R594K       | 28 ± 4 \(\mu\)m         | 32 ± 3 \(\mu\)m      |

FIGURE 3. Leu\(^{556}\) and Trp\(^{586}\) are required for the bisanandrolaphide-induced activation of TRPV4. A and B, effect of stimulation with 10 \(\mu\)m BAA on [Ca\(^{2+}\)]

in wild type TRPV4 (A), Y556A,W586A (A), Y556A (B), and L584M (B) transfected HEK cells. C, dose response of BAA on peak \(\Delta [Ca^{2+}]_i\)

in wild type TRPV4 (●), W586A (●), and Y556A (○) transfected HEK cells. For every condition, \(n>16\) in at least three independent experiments.

FIGURE 4. Effect of S3–S4 mutagenesis on activation of TRPV4 by heat. A, basal [Ca\(^{2+}\)] in nontransfected (NT), wild type TRPV4, and TRPV4 mutant transfected HEK cells. YW-6AA-, Y556A-, W586A-, WM-6AA, WM-6AA, W586A-, YM-6AA, Y556A, M587A, B, average increase in [Ca\(^{2+}\)] in response to moderate warmth (42 °C) application. For every condition, \(n>26\) in at least three independent recordings. * significant differences when compared with cells expressing wild type TRPV4.

Trp\(^{586}\), or Tyr\(^{556}\) was not due to an effect on channel expression/targeting. First, cell surface biotinylation experiments confirmed that all these three mutant channels are expressed in the plasma membrane (supplemental Fig. 1). Second, and more importantly, mutants L584M, W586A, Y556A, Y556A,W586A, Y556A,M587A, and W586A,M587A all responded to hyposmotic cell swelling and to exogenously applied AA (10 \(\mu\)m) or 5,6-EET (500 \(\mu\)m), and the amplitudes of the [Ca\(^{2+}\)] responses and inward currents were similar to those of wild type TRPV4 (Fig. 5). Note that all mutants displayed similar IV relationships as WT-TRPV4, indicating that the pore properties are unaltered (supplemental Fig. 2). Taken together, these results indicate that residues Leu\(^{556}\) and Trp\(^{586}\) at the C-terminal part of TM4 are selectively involved in activation by 4α-PDD, BAA, and heat.

 Activation of TRPV4 by Different 4α-Phorbol Esters—The finding that residues both at the intracellular end of TM3 (Tyr\(^{556}\)) and at the extracellular part of TM4 (Leu\(^{556}\) and Trp\(^{586}\)) are crucial for 4α-PDD activation may suggest that 4α-PDD interacts with TRPV4 in a region between TM3 and TM4. This raises the question how 4α-PDD is oriented with respect to the channel. To approach this question, we examined how the length of the aliphatic side chains attached to the 4α-phorbol moiety determines TRPV4 activation. We compared potencies of 4α-PDBu, 4α-PMA, 4α-PDA, and 4α- phorbol (Fig. 6A) on activation of wild type and mutant TRPV4 channels. In the presence of extracellular Ca\(^{2+}\), application of all these 4α-phorbol derivatives to HEK cells transfected with WT TRPV4 resulted in dose-dependent increases of [Ca\(^{2+}\)]

albeit with lower potencies (i.e. higher EC\(_{50}\) values) than for 4α-PDD (Fig. 6B). No [Ca\(^{2+}\)] increase was observed in nontransfected cells or when extracellular Ca\(^{2+}\) was omitted (data not shown). Note that the steepness of the dose-response curves also differed significantly between the 4α-phorbols, reflected in the variation in \(n_H\) values (4.5 for 4α-PDD, 1.8 for 4α-PMA, and 0.9 for 4α-PDBu). Although in theory, differences in \(n_H\) may be indicative of altered cooperativity of ligand binding and/or altered number of binding sites, we do not think that we can make such conclusions from our data. Indeed, the steepness of the dose-response curves is not only determined by the channel-ligand interaction but also by factors such as Ca\(^{2+}\) dye saturation and the time course of channel desensitization.

Application of 4α-PDBu to wild type TRPV4 transfected HEK cells caused activation of typical outwardly rectifying currents (additional currents at -80 mV, \(\Delta I_{-80\, mV}\) -178 ± 34 pA; \(n=8\)) (Fig. 6F). Similar currents were also evoked by 4α-PDA (\(\Delta I_{-80\, mV}\) -142 ± 13 pA; \(n=4\)) and 4α-phorbol (\(\Delta I_{-80\, mV}\) -185 ± 38 pA; \(n=5\)) (Fig. 6, D and E), whereas no current increases were observed in nontransfected HEK cells. Patch clamp experiments showing TRPV4 activation by 4α-PMA have been reported in previous work (14).

Since TM3–4 residues participate in 4α-PDD sensitivity of TRPV4, we tested the potency of the different 4α-phorbols to activate Y556A and W586A mutants. In comparison with WT TRPV4, mutant Y556A had a lower sensitivity to 4α-PDD, 4α-PDBu, and 4α-PMA (Fig. 7, A and C, and Table 2). However, 4α-PDA and 4α-phorbol activated WT TRPV4 and the Y556A mutant with similar potency (Fig. 7C and Table 2), suggesting that Tyr\(^{556}\) is only involved in channel activation by 4α-phorbols with longer aliphatic chains. In contrast, mutant W586A not only displayed a significantly reduced sensitivity to both 4α-PDD and 4α-PDBu but was also fully unresponsive to 4α-PDA, 4α-PMA and 4α-phorbol (Fig. 7, D and F, and Table 2). This indicates that this residue at the extracellular part of TM4 is involved in channel activation by all 4α-phorbols.

Mutations to the C-terminal Part of TM4 Affect General Activation of TRPV4—In contrast to the above described mutants in the central part of TM4, mutating residues Tyr\(^{591}\) and Arg\(^{594}\) in the C-terminal part of TM4 did not reduce the basal channel activity as evidenced by the elevated [Ca\(^{2+}\)] levels in HEK cells expressing the Y591A and R594A mutants. In fact, the basal
[Ca\(^{2+}\)]_i level in cells expressing the Y591A was significantly higher than in those expressing WT TRPV4 (Fig. 8A), suggesting a higher level of basal activity. This was confirmed in whole-cell current measurements showing robust basal currents (mean amplitude at −80 mV for nontransfected ~39 ± 8 pA; WT TRPV4 ~75 ± 7 pA and Y591A transfected HEK cells ~191 ± 25 pA and p < 0.01) for Y591A, which were blocked in a voltage-dependent manner by the TRPV4 blocker Ruthenium Red (2 μM) (Fig. 8B). The increases in [Ca\(^{2+}\)]_i and whole-cell current measured in response to all tested stimuli (4α-PDD, heat, cell swelling, AA, and 5,6-EET) were always lower in cells expressing Y591A than in those expressing WT TRPV4 (Figs. 8, C, D, and G). These lower responses are most likely a consequence of the higher basal activity of the Y591A mutant as the absolute [Ca\(^{2+}\)]_i at the peak of the response to these different stimuli was not significantly different from those obtained with WT TRPV4 (Fig. 8H).

DISCUSSION

Several members of the TRP superfamily act as chemosensors, activating in response to a multitude of endogenous and exogenous ligands. At present, relatively little is known about how these ligands interact with TRP channels and how this interaction regulates channel gating. In this study, we provide evidence suggesting that the TM3–4 region of TRPV4 forms an important site for channel activation by phorbol esters. In particular, we found that mutations at positions Tyr556 in TM3 or Leu584 and Trp586 in TM4 strongly affected channel activation by the most potent and selective agonist 4α-PDD. Several possible mechanisms could explain a reduced sensitivity by 4α-PDD or BAA in these TRPV4 mutants. Firstly, these mutants may endure a trafficking problem to the plasma membrane. However, this possibility can be excluded given that these mutants could still be normally activated by hypotonic cell swelling, AA, and 5,6-EET and that they could be detected as biotinylated proteins at the cell surface (supplemental Fig. 1). Secondly, 4α-PDD binding may occur through an unknown accessory 4α-PDD-binding protein. The effects of mutations in TM3–4 may then reflect an altered interaction between channel and accessory protein. Although we cannot formally exclude this possibility, we consider it as relatively unlikely. If we accept that TRPV4 is structurally related to six TM K⁺ channels, the crucial TM3–4 residues point toward each other at the center of the TM1–4 region, where interactions with accessory proteins are unlikely to occur. Thirdly, these residues
may form the structural element necessary for the transduction of the agonist-binding signal to the opening of the pore. An indication against such a mechanism is that opening of the mutant channels is normal upon stimulation with other ligands, such as 5,6-EET or, in the case of Y556A, BAA. Therefore, at present, we prefer the fourth possible mechanism, namely that these residues in TM3 and TM4 are crucial for interaction with 4α-PDD and possibly part of 4α-PDD-binding site.

To obtain better insight in the activation mechanism of TRPV4 by 4α-PDD, we compared the potency of 4α-phorbol and 4α-phorbol esters with different aliphatic side chains. For WT TRPV4, we found an inverse correlation between the length of the aliphatic side chain and the EC50 value for channel activation (Fig. 6C), suggesting that a hydrophobic side chain enhances the interaction with the binding site. Interestingly, the Y556A mutant displayed a reduced sensitivity to stimulation by 4α-phorbol esters with longer aliphatic side chains, such as 4α-PDD, 4α-PMA, and 4α-PDBu, whereas responses to 4α-PDA and 4α-phorbol remained unaffected. This suggests that Tyr556 is involved in the interaction between TRPV4 and the aliphatic part of 4α-phorbol esters. In contrast, mutating Trp586 impaired channel activation by all 4α-phorbols, suggesting that this residue interacts with the 4α-phorbol moiety of 4α-PDD.

Based on these data, and in analogy to what has been proposed for the interaction between capsaicin and TRPV1 (19, 20), we propose a tentative model in which the pocket between TM3 and TM4 constitutes a binding site for 4α-phorbol (Fig. 10). In this model, 4α-PDD is positioned such that the aliphatic side chains point toward the cytosol, where they can interact with Tyr556, whereas the 4α-phorbol moiety is positioned closer to the extracellular side of the plasma membrane, where it can interact with residues Trp586 and Leu584. The higher EC50 values for double mutants Y556A,M587A and W586A,M587A when compared with the single mutants Y556A and W586A, is suggestive of an indirect role of Met587. In the interaction with 4α-phorbols, although more evidence is required to establish this point. Clearly, structural data will be required to validate the location of the binding sites in TRPV4 for 4α-PDD and related compounds. The orientation of the ligand between TM3–4 of TRPV4 is similar to a putative model for a capsaicin-binding site of TRPV1 proposed by Gavva et al. (20) in which the aliphatic chain of capsaicin points toward the intracellular side of the membrane, whereas the vanilloid part is positioned closer to the extracellular side of the plasma membrane. Similarly, a recent study provides evidence for a direct interaction.

FIGURE 6. Activation potency of different 4α-phorbol esters. A, molecular structure of different 4α-phorbol esters. B, dose-response curve of the average increase in [Ca2+]i, in wild type TRPV4 transfected HEK cells after stimulation with 4α-phorbol (□), 4α-PMA (●), 4α-PDBu (○), and 4α-PDD (▲). C, activation potency versus length aliphatic side chain. EC50 values are an approximation obtained by extrapolation of the dose-response fits. D–G, time course of whole-cell currents at −80 and +80 mV in wild type TRPV4-expressing HEK cells on stimulation with 50 μM 4α-phorbol (D), 50 μM 4α-PDA (E), 10 μM 4α-PDBu (F), and 1 μM 4α-PDD (G).

FIGURE 7. Activation potency of different 4α-phorbol esters after TM3–4 mutagenesis. A and B, dose-response curve of 4α-PDBu on the average increase in [Ca2+]i in wild type TRPV4- (□) and Y556A- (●) and W586A- (▲) expressing HEK cells. C, dose-response curve of 4α-phorbol on peak [Ca2+]i in wild type TRPV4- (□) and Y556A- (●) and W586A- (▲) expressing HEK cells. D, effect of added 4α-phorbol (100 μM) on [Ca2+]i in W586A transfected HEK cells. E, dose-response curve of 4α-PMA on the average increase in [Ca2+]i in wild type TRPV4- (□) and Y556A- (●). F, effect of added 4α-PMA (50 μM) on [Ca2+]i in W586A transfected HEK cells. For every condition, n > 23, data obtained from at least three independent experiments were pooled.
between the cooling agent menthol and the cold- and menthol-sensitive channel TRPM8 (28). It should be stressed that although these models adequately explain the functional results, they are still speculative and require additional biochemical and structural information for validation.

At room temperature (~25 °C), TRPV4 exhibits a basal activity that is manifested by an increased basal [Ca^{2+}], level in TRPV4-overexpressing cells (17) (Fig. 4A). Interestingly, cells expressing the different mutants with reduced 4α-PDD did not exhibit such elevated basal [Ca^{2+}], levels and no longer responded to a heat stimulus. In contrast to other heat-sensitive TRPV channels, which are directly activated by temperature changes, heat activation of TRPV4 does not occur in cell-free inside-out patches, which may indicate that soluble messengers play a role in this process (9, 13, 27). One possibility would be that increases in temperature lead to the generation of a ligand activator of TRPV4. Our finding that several mutations in the TM3–4 region equally affect sensitivity to heat and 4α-PDD would then suggest that the heat-induced messenger and 4α-PDD have overlapping binding sites. We reasoned that sphingomyelin metabolites, which are generated upon heat shock and serve as messengers of this stress condition (29), could play the role of heat-inducible TRPV4 agonists, but direct application of sphingosine-1-phosphate, sphingosine, or ceramide did not evoke any response in TRPV4-expressing cells (data not shown). Moreover, we found that mutant R594A was unresponsive to all tested TRPV4 activation stimuli with the
exception of heat stimulation. Thus, at this point, the mecha-
nism underlying heat activation of TRPV4 remains elusive.

Two conserved residues located closer to the C terminus of
TM4 (Fig. 10) appear to play a more general role in activation
of TRPV4. Mutation of Tyr591 to an alanine resulted in a channel
with high basal activity, as evidenced by the increased basal
[Ca\(^{2+}\)], and robust unstimulated whole-cell currents in cells
expressing Y591A. This mutant channel exhibited significant
but reduced responses to all tested stimuli, which most likely
reflects the already elevated basal activity of the mutant chan-
nel. Mutating the highly conserved basic residue Arg594 to ala-
ine or glutamine impaired responsiveness of the TRPV4 chan-
nel. Mutating the highly conserved basic residue Arg594 to alan-
ine or glutamine impaired responsiveness of the TRPV4 chan-
nel to 4α-PDD, cell swelling, AA, and 5,6-EET, whereas
activation by moderate heat remains unchanged. Substituting a
lysine for Arg594 yielded a mutant channel that exhibited simi-
lar responses as WT TRPV4, suggesting that a charged residue
at this position is required for the transduction of chemical
stimuli.

In conclusion, we propose that the TM3–4 region plays a
central role in the activation of TRPV4 by thermal and chemical
stimuli and may form a possible interaction site for BAA and
4α-phorbols.

Acknowledgment—We thank Prof. D. Clapham (Children’s Hospital,
Harvard Medical School, Boston, MA), for providing bisandrogra-
pholide A.

REFERENCES

1. Clapham, D. E., Montell, C., Schultz, G., and Julius, D. (2003) Pharmacol.
Rev. 55, 591–596
2. Vriens, J., Owsianik, G., Voets, T., Droogmans, G., and Nilius, B. (2004)
Pflugers Arch. Eur. J. Physiol. 449, 213–226
3. Nilius, B., Vriens, J., Prenen, J., Droogmans, G., and Voets, T. (2004) Am. J.
Physiol. 286, C195–C205
4. Nilius, B., and Voets, T. (2005) Pflugers Arch. Eur. J. Physiol. 451, 1–10
5. Pedersen, S. F., Owsianik, G., and Nilius, B. (2005) Cell Calcium 38, 233–252
6. Owsianik, G., D’Hoedt, D., Voets, T., and Nilius, B. (2006) Rev. Physiol.
Biochem. Pharmacol. 156, 61–90
7. Owsianik, G., Talavera, K., Voets, T., and Nilius, B. (2006) Annu. Rev.
Physiol. 68, 685–717
8. Strotmann, R., Harteneck, C., Nunnenmacher, K., Schultz, G., and Plant,
T. D. (2000) Nat. Cell Biol. 2, 695–702
9. Guler, A. D., Lee, H., Iida, T., Shimizu, I., Tominaga, M., and Caterina, M.
(2002) J. Neurosci. 22, 6408–6414
10. Wissenbach, U., Bodding, M., Freichel, M., and Flockerzi, V. (2000) FEBS Lett.
485, 127–134
11. Liedtke, W., Choe, Y., Marti-Renom, M. A., Bell, A. M., Denis, C. S., Sali,
A., Hudspeth, A. J., Friedman, J. M., and Heller, S. (2000) Cell 103, 525–535
12. Nilius, B., Prenen, J., Wissenbach, U., Bodding, M., and Droogmans, G.
(2001) Pflugers Arch. Eur. J. Physiol. 443, 227–233
13. Watanabe, H., Vriens, J., Suh, S. H., Benham, C. D., Droogmans, G., and
Nilius, B. (2002) J. Biol. Chem. 277, 47044–47051
14. Watanabe, H., Davis, J. B., Smart, D., Jerman, J. C., Smith, G. D., Hayes, P.,
Vriens, J., Cairns, W., Wissenbach, U., Prenen, J., Flockerzi, V., Droog-
mans, G., Benham, C. D., and Nilius, B. (2002) J. Biol. Chem. 277, 13569–13577
15. Smith, P. L., Maloney, K. N., Pothen, R. G., Clardy, J., and Clapham, D. E.
(2006) J. Biol. Chem. 281, 29897–29904
16. Watanabe, H., Vriens, J., Prenen, J., Droogmans, G., Voets, T., and Nilius,
B. (2003) Nature 424, 434–438
17. Vriens, J., Watanabe, H., Janssens, A., Droogmans, G., Voets, T., and
Nilius, B. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 396–401
18. Vriens, J., Owsianik, G., Fisslthaler, B., Suzuki, M., Janssens, A., Voets, T.,
Morisseau, C., Hammock, B. D., Fleming, I., Busse, R., and Nilius, B. (2005)
Circ. Res. 97, 908–915
19. Jordt, S. E., and Julius, D. (2002) Cell 108, 421–430
20. Gavva, N. R., Klionsky, L., Qu, Y., Shi, L., Tamir, R., Edenson, S., Zhang,
T. I., Viswanadhan, V. N., Toth, A., Pearce, L. V., Vainerah, T. W., Por-
reca, F., Blumberg, P. M., Lile, J., Sun, Y., Wild, K., Louis, J. C., and Treanor,
J. J. (2004) J. Biol. Chem. 279, 20283–20295
21. Vlahova, V., Teisinger, J., Susankaova, K., Lyfenko, A., Ettrich, R., and
Vyklicky, L. (2003) J. Neurosci. 23, 1340–1350
22. Jung, J., Lee, S. Y., Hwang, S. W., Cho, H., Shin, J., Kang, Y. S., Kim, S., and
Oh, U. (2002) J. Biol. Chem. 277, 44448–44454
23. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989)
Gene (Amst.) 77, 51–59
24. Vriens, J., Janssens, A., Prenen, J., Nilius, B., and Droogmans, G. (2003) Cell
Calcium 36, 19–28
25. Paltauf-Dobargaynska, I., and Graier, W. F. (1997) Cell Calcium 21, 43–51
26. Strotmann, R., Schultz, G., and Plant, T. D. (2003) J. Biol. Chem. 278,
26541–26549
27. Voets, T., Talavera, K., Owsianik, G., and Nilius, B. (2005) Nat. Chem. Biol.
1, 85–92
28. Voets, T., Owsianik, G., Janssens, A., Talavera, K., and Nilius, B. (2007)
Nat. Chem. Biol. 3, 174–182
29. Hannun, Y. A., and Luberto, C. (2000) Trends Cell Biol. 10, 73–80