The Integrity of the TRP Domain Is Pivotal for Correct TRPV1 Channel Gating

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ABSTRACT Transient receptor potential vanilloid subtype I (TRPV1) is a thermosensory ion channel that is also gated by chemical substances such as vanilloids. Adjacent to the channel gate, this polymodal thermoTRP channel displays a TRP domain, referred to as AD1, that plays a role in subunit association and channel gating. Previous studies have shown that swapping the AD1 in TRPV1 with the cognate from the TRPV2 channel (AD2) reduces protein expression and produces a nonfunctional chimeric channel (TRPV1-AD2). Here, we used a stepwise, sequential, cumulative site-directed mutagenesis approach, based on rebuilding the AD1 domain in the TRPV1-AD2 chimera, to unveil the minimum number of amino acids needed to restore protein expression and polymodal channel activity. Unexpectedly, we found that virtually full restitution of the AD1 sequence is required to reinstate channel expression and responses to capsaicin, temperature, and voltage. This strategy identified E692, R701, and T704 in the TRP domain as important for TRPV1 activity. Even conservative mutagenesis at these sites (E692D/R701K/T704S) impaired channel expression and abolished TRPV1 activity. However, the sole mutation of these positions in the TRPV1-AD2 chimera (D692E/K701R/S704T) was not sufficient to rescue channel gating, implying that other residues in the TRP domain are necessary to endow activity to TRPV1-AD2. A biophysical analysis of a functional chimera suggested that mutations in the TRP domain raised the energetics of channel gating by altering the coupling of stimuli sensing and pore opening. These findings indicate that inter- and/or intrasubunit interactions in the TRP domain are essential for correct TRPV1 gating.

INTRODUCTION

TRPV1 is a polymodal receptor that is gated by vanilloid compounds such as capsaicin and resiniferatoxin, acidic extracellular pH, strong membrane depolarization, and temperatures \( \geq 42^\circ \text{C} \) at the membrane resting potential (1,2). This receptor displays high temperature sensitivity, with \( Q_{10} \geq 20 \) (3–5). Polymodal gating implies the action of different sensors that can work independently or in concert to activate the channel (6–9). The intricacy of TRPV1 polymodal gating is further enhanced by algesic-induced TRPV1 potentiation, which increases the sensitivity to the activating stimuli (10–14).

A functional TRPV1 channel is a homotetramer of subunits assembled around a central aqueous pore (1,15). Each subunit exhibits three distinct domains: 1) a six-transmembrane segment region (S1–S6) that assembles the aqueous pore, the capsaicin-binding site, and the putative voltage sensor (16); 2) a cytosolic N-terminus containing ankyrin domains involved in protein-protein interactions with components of the receptor signalplex (17,18); and 3) a cytosolic C-terminus that harbors a TRP domain, phosphoinositide-interacting sites, and consensus regions for protein kinases and phosphatases (15,19–21).

The TRP domain, a segment adjacent to the C-terminus of S6 (see Fig. 1 A), acts as an association domain (AD) for subunit tetramerization and contributes to the allosteric coupling of stimuli sensing and gate opening (21–24), as evidenced by several findings: 1) deletion of this region in TRPV1 impairs channel expression in the cell surface (24); 2) replacement of the TRP domain of TRPV1 with the cognate region of other TRPV channels (TRPV2-TRPV6) partially affects receptor synthesis and delivery to the cell surface, and notably impacts the channel functionality (21); 3) amino acids I696 and W697 are pivotal for voltage, capsaicin, and temperature gating (22,23); and 4) synthetic peptides patterned after the N-terminus of this domain act as allosteric antagonists when delivered intracellularly or tethered to the plasma membrane, blocking all modes of channel gating (25). Notably, a structural TRPV1 model at 3.4 Å, inferred from cryo-electron microscopy (cryo-EM) images (26,27), showed that the TRP domain of TRPV1 is an \( \alpha \)-helix that runs parallel to the inner leaflet of the membrane and interacts with several intracellular domains of receptor subunits. This structural arrangement suggests that the entire TRP domain is essential for channel function.
Here, we addressed this issue and investigated the structural requirements of the TRP domain that are compatible with TRPV1 channel gating. We employed a stepwise, sequential, cumulative site-directed mutagenesis approach to rebuild the AD1 in a nonfunctional TRPV1-AD2 chimera (containing the AD of TRPV2) (see Fig. 1 B) as a means to unveil the minimum number of amino acids that are required for channel activity in the nonfunctional chimeric channel. Unpredictably, we found that a virtually intact AD1 domain is necessary to improve protein expression and reinstate TRPV1 channel gating. Among the 19 chimeras we designed, only those that exhibited a high sequence identity to AD1 gradually restored the responses to voltage, temperature, and capsaicin. Furthermore, our results identified E692, R701, and T704 in the TRP domain of TRPV1 as important molecular determinants of allosteric channel activation. Collectively, our findings imply that the integrity of the TRP domain in TRPV1 is important for fine-tuning stimuli sensing and gate opening, and that even conservative alterations in this domain negatively affect channel expression and function. Our results are consistent with the high sequence conservation of this domain in TRPV1 channels among mammalian species (see Fig. 1 C).

MATERIALS AND METHODS

TRPV1 and TRPV1-AD2 receptor mutagenesis

We performed mutagenesis on rat TRPV1 and TRPV1-AD2 receptors using Pfu Turbo DNA polymerase (QuickChange II, Agilent Technologies, Santa Clara, CA) according to the manufacturer’s recommendations. Mutant channels were confirmed by DNA sequencing.

Cell culture and transfection

HEK293 kidney epithelial cells were cultured in Dulbecco’s modified Eagle’s medium-Glutamax supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin solution as described previously (22,23). Isolated HEK293 cells were transfected with DNA encoding the TRPV1 and mutant channels via calcium phosphate precipitation or serum and 1% penicillin/streptomycin solution as described previously (22,23). HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium-Glutamax supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin solution as described previously (22,23). HEK293 cells were cultured in Dulbecco’s medium-Glutamax supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin solution as described previously (22,23). Isolated HEK293 cells were transfected with DNA encoding the TRPV1 and mutant channels via calcium phosphate precipitation or Lipofectamine 2000 (Life Technologies, Madrid, Spain) according to the manufacturer’s recommendations. Transfected cells were used 24–48 h after transfection.

Cell-surface protein biotinylation

Biotinylation of surface proteins was carried out with sulfo-NHS-LC-biotin (Thermo Fisher Scientific) as described previously (22). Proteins were separated in 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes for western immunoblot analysis using polyclonal anti-TRPV1 (1:30,000, rabbit; Alomone Labs) and anti-actin (1:10,000, rabbit; Sigma-Aldrich) overnight at 4°C. After extensive washing, the membranes were exposed to anti-rabbit immunoglobulin G peroxidase conjugate (1:50,000; Sigma-Aldrich) for 1 h at room temperature and detected using the chemiluminescent detection reagent ECL Select (GE Healthcare). Immunoblots were digitized and quantified with TotalLab Quant software.

Calcium fluorography screening assay

Cells were seeded in a black 96-well plate with a clear, flat bottom at 10,000–20,000 cells/well and incubated with Fluoro-NW in 100 μl of Hank’s balanced salt solution (20 mM HEPES, pH 7.4) in the presence of 2.5 mM probenecid for 45 min at 37°C and 10 min at room temperature. The Fluoro-4 fluorescence signal (excitation at 494 nm and emission at 516 nm) was measured with a POLARStar Omega microplate reader (BMG Labtech) during 18 cycles. Channel activity was activated with 100 μM of capsaicin injected during the fifth cycle. The capsaicin response was estimated from the area under the curve (AUC) between the 5th and 18th cycles.

Patch-clamp measurements

Whole-cell patch-clamp recordings were made in HEK293 cells as previously described (22,23). For electrophysiological recordings, the pipette solution contained (in mM) 150 NaCl, 3 MgCl2, 5 EGTA, and 10 HEPES, pH 7.2 with CsOH, and the extracellular solution contained (in mM) 150 NaCl, 6 CaCl2, 1 MgCl2, 1.5 CaCl2, 10 glucose, and 10 HEPES, pH 7.4 with NaOH. Data were sampled at 10 kHz (EPC10 with Pulse software; HEKA Elektronik) and low-pass filtered at 3 kHz for analysis (PulseFit 8.54; HEKA Elektronik). The series resistance was usually <10 MΩ and to minimize voltage errors was compensated to 70–90%. Recordings with leak currents of >100 pA or series resistance of >10 MΩ were discarded.

Conductance-voltage (G-V) curves were obtained from the tail currents. Normalized G-V curves were fitted to the Boltzmann equation:

$$\frac{G}{G_{\text{max}}} = \frac{G_{\text{min}}}{G_{\text{max}}} + \left(1 + e^{\frac{V - V_{\text{0.5}}}{\Delta G_{\text{0.5}}}}\right)^{-1},$$

where $G_{\text{max}}$ is the true maximal conductance of the channel species obtained in the presence of 10 μM of capsaicin for wild-type channels and 100 μM of capsaicin for AD2-18 and AD2-19 chimeras, $G_{\text{min}}$ is the minimal conductance at hyperpolarized potentials, $V_{0.5}$ is the voltage required to activate the half-maximal conductance, and $\Delta G_{0.5}$ is the gating valence. For voltage-dependent gating, the free-energy difference between the closed and open states at 0 mV and 22°C for a two-state model ($\Delta G_{\text{0.5}}$) was calculated as described previously (22,23).

Temperature activation

Temperature jumps were produced using a single emitter laser diode as the heat source (28). The conductance-temperature (G-(1/T)) curves were obtained by converting the maximal current values from the temperature step protocol to conductance using the relation $G = I/(V - V_{\text{R}})$. Normalized $G-(1/T)$ curves were fitted to the Boltzmann equation:

$$\frac{G}{G_{\text{max}}} = \frac{G_{\text{min}}}{G_{\text{max}}} + \left(1 + e^{\frac{T - T_{0.5}}{\Delta H}}\right)^{-1},$$

where $T_{0.5}$ is the temperature required to activate the half-maximal conductance, $\Delta H$ is the activation enthalpy, and $R$ is the universal gas constant (5). Thermodinamic parameters for the temperature dependence of channel opening were calculated using the van ‘t Hoff equation:

$$\ln K_{\text{eq}} = -\frac{\Delta H}{R} \frac{1}{T} + \frac{\Delta S}{R},$$

where $\Delta H$ and $\Delta S$ correspond to enthalpy (cal/mol) and entropy (cal mol$^{-1}$K$^{-1}$) of the gating process between closed and open states, $T$ is the
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temperature applied in every step, \( R \) is the gas constant, and \( K_{eq} \) is the equilibrium constant of the channel-opening process obtained from Yao et al. (5):

\[
K_{eq} = \frac{G/G_{\text{max}}}{1 - \left( G/G_{\text{max}} \right)}.
\]

The free-energy difference between the closed and open states was calculated from \( \Delta G = RT \ln(K_{eq}) \).

Temperature sensitivity was estimated with the \( Q_{10} \) parameter from macroscopic currents, obtained using

\[
\log \left( \frac{I}{I_{\text{max}}} \right) = -(10 \times \log Q_{10}) \times \left( \frac{1}{T} \right),
\]

where \( I \) represents the current obtained at temperature \( T \) (°K), and \( I_{\text{max}} \) denotes the current obtained at a 333°K.

Data processing and statistics

Data were analyzed using PulseFit 8.11 (HEKA Elektronik), pCLAMP 10.3 (Axon Instruments, Foster City, CA), or custom-made software (Q-studio). GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) was used for statistics. All data were expressed as the mean ± SE, and \( n \) = the number of cells tested for electrophysiological data or the number of experiments conducted for biochemical data. Statistical analysis was performed by using one-way ANOVA or Student’s test as indicated, and \( p < 0.05 \) was taken as the minimum level of significance.

Molecular modeling

We modeled a molecular model for TRPV1-AD2 chimeras using the structures of the TRPV1 ion channel in the closed (PDB code 3J5P) and open (PDB code 3J5R) states as determined by EM at 3.4 Å resolution (26,27). Modeling was carried out as previously described (22,29,30). Visualization and editing of the molecules were done with Yasara (31)(http://www.yasara.org). Briefly, the modeling was performed according to the standard modeling protocol implemented in Yasara (v14.6.23). In this process, only the small loops were modeled, avoiding the modeling of S2-S3 or S5-pore helix loops. After construction, optimization, and fine-tuning of the side chains, all newly modeled parts were subjected to a combination of steepest descent and simulated annealing minimization, keeping the backbone atoms fixed to avoid molecule damage. Finally, a short, fully unrestrained simulated annealing minimization was run for the entire model.

The protein design algorithm and force field FoldX from CRG (http://foldx.crg.es) were used for protein mutagenesis and theoretical energy measurements (32). The interaction energy between different parts of the molecule was calculated by unfolding the selected regions and determining the stability energy of the separated molecules, and then subtracting the sum of individual energies from the global energy of the complex. In addition, the model was evaluated using PROCHECK to show the residues in the allowed regions of the Ramachandran plots (33). The final molecular graphic representations were created using PyMOL v1.4.1 (http://www.pymol.org/).

RESULTS

Reconstruction of the AD1 domain in a nonfunctional TRPV1-AD2 chimera

Previously, replacement of AD1 (aa 682–722; Fig. 1 A) in rat TRPV1 by the cognate AD2 region in TRPV2 was shown to produce a nonfunctional TRPV1-AD2 chimeric channel (21). To unveil the molecular determinants required to restore channel activity to this chimera, we designed a stepwise, sequential, cumulative site-directed mutagenesis approach to rebuild the AD1 domain in the TRPV1-AD2 chimera (Fig. 1 B). Our strategy was to progressively incorporate the different AD1 residues in TRPV1-AD2 with the following priority: 1) amino acid insertions, along with Ser-683 to Gly, which was considered important for its proximity to the channel gate (Ile-679); 2) mutation of distantly related amino acids; 3) mutation of amino acids with similar physicochemical properties; and 4) mutation of the most conserved/similar amino acids. We obtained a total of 20 mutant species, referred to as AD2-1 to AD2-20. AD2-20 displays a reinstated AD1 domain, i.e., a

\[
\begin{align*}
K_{eq} & = \frac{G/G_{\text{max}}}{1 - \left( G/G_{\text{max}} \right)} \quad (\text{RT} \ln(K_{eq})) \\
\log \left( \frac{I}{I_{\text{max}}} \right) & = -(10 \times \log Q_{10}) \times \left( \frac{1}{T} \right),
\end{align*}
\]

**FIGURE 1** Schematic representation of the step reconstruction of the AD1 domain in the TRPV1-AD2 chimera by mutagenesis of the AD2 domain. (A) The original chimera (TRPV1-AD2) corresponds to rat TRPV1, with its TRP domain (orange) replaced by the cognate part of human TRPV2 (black) (21). Residue numbers correspond to rat TRPV1. (B) The chimera TRPV1-AD2 is cumulative and sequentially mutated, rescuing the AD1 sequence of TRPV1. In this process, 19 mutants (AD2-1 to AD2-19) were generated. AD2-20 fully reinstated the AD1 sequence (TRPV1-AD1), thus corresponding to the wild-type. The mutated residues in each step are highlighted in red, the conserved amino acids between AD1 and AD2 are in black, and the nonconserved residues are in blue. (C) Sequence alignment of the TRP domains of TRPV1 orthologs from rat, human, mouse, rabbit, guinea pig, naked mole rat, and dog.
wild-type channel (Fig. 1 B). Note that the AD1 domain is highly conserved among mammalian species (Fig. 1 C).

We evaluated the functionality of the mutants by Ca\textsuperscript{2+}–fluorography using a 96-well plated reader. Fluor4-loaded HEK293 cells expressing the different mutant channels were stimulated with 100 \mu M of capsaicin and the Ca\textsuperscript{2+} response was recorded (Fig. 2 A). A capsaicin-induced increase in cytosolic Ca\textsuperscript{2+} was clearly observed in HEK293 cells that expressed the chimeras AD2-18 and AD2-19, and reinstated the wild-type (AD2-20) (Fig. 2, A and B). All other mutant species displayed nonsignificant fluorescence changes as compared with TRPV1-AD2 or mock-transfected cells (Fig. 2, A and B), except for AD2-12 and AD2-13, which displayed barely detectable vanilloid responses. Channel activity started to be noticeable and significant in AD2-18, increased in AD2-19, and was identical to that of the wild-type in AD2-20 (TRPV1-AD1). Mutations that led to the recovery of channel activity involved conservative changes of K701R in AD2-18, D692E in AD2-19, and S704T/E707D to reproduce AD1 (AD2-20) (Fig. 1 B). As expected, cumulative and conservative mutation of the cognate residues E692, R701, and T704 in TRPV1 (TRPV1(E692D/R701K/T704S)) progressively led to full abrogation of channel function (Fig. 2 C). This result substantiates that these residues are pivotal for channel gating, and corroborate the role of R701, which was previously implicated as a molecular determinant of TRPV1 channel gating (23).

A question that arises from our strategy is whether the restored TRPV1 functionality was due to the reinstated AD1 sequence or just to mutation of the amino acids changed last, namely, D692, K701, S704, and E707. To investigate whether mutation of only these positions in the TRPV1-AD2 chimera was sufficient to reestablish channel activity, we sequentially mutated these positions in TRPV1-AD2 (Fig. 2 C). As can be seen in the figure, direct mutation of these sites in the TRPV1-AD2 chimera (TRPV1-AD2 (D692E/K701R/S704T/E707)) did not restore the capsaicin responses (Fig. 2 C). This result implies that other positions in the AD2 domain need to be mutated to the cognate AD1 amino acid for channel functionality, suggesting that a virtually integral AD1 domain is required for TRPV1 channel activity.

**Restoration of the AD1 sequence in TRPV1-AD2 increases receptor expression and reestablishes N-glycosylation**

We next compared the expression levels of the nonfunctional TRPV1-AD2, AD2-4, and AD2-17 chimeras, as well as the functional AD2-18 and AD2-19 chimeras, with those of the wild-type channels (TRPV1 and AD2-20) (Fig. 3). All chimeras displayed a reduced protein expression as compared with TRPV1 (Fig. 3 A). Relative quantitation of the total protein levels clearly displayed increased channel expression as the AD1 domain was reinstated.

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**FIGURE 2.** TRPV1 activity recovery from the nonfunctional chimera TRPV1-AD2. (A) Change in the Ca\textsuperscript{2+}–dependent fluorescence of transfected HEK cells as a function of time, before and after exposure to vehicle (0.1\% DMSO) (left panel) or 100 \mu M of capsaicin (right panel) at 25°C. Traces represent the average in fluorescence changes from all experiments performed for TRPV1 and some representative TRPV1-AD2 mutants. The arrows indicate the onset of vehicle and capsaicin exposure. (B) Normalized values of fluorescence obtained from the AUC for TRPV1, mock transfected cells, AD2 chimeric mutants, and AD2 point mutants. (C) Normalized AUC values for TRPV1 wild-type and mutants, and TRPV1-AD2 mutants. Data are shown as the mean ± SE, n ≥ 4. *p < 0.05, **p < 0.01, and ***p < 0.005. Statistical analysis corresponds to one-way ANOVA comparing chimeras with wild-type channels. To see this figure in color, go online.
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FIGURE 3 Mutations within the TRP domain affect the receptor expression level. (A) TRPV1 species were transiently expressed in HEK293 cells. Western immunoblotting was done using a polyclonal anti-TRPV1 antibody. Whole-cell and avidin-purified extracts were separated in 10% SDS-PAGE gels, and immunoblots were visualized with the ECL system. G, N-type glycosylated; NG, non-N-type glycosylated. (B) Quantification obtained with TOTAL LAB software and expressed as the total amount of protein (light gray) and surface/total protein ratio (dark gray) of the bands shown in (A). Actin was used as a loading control. Mutants were chosen as representative of the TRP domain recovery approach. Data are depicted as the mean ± SE, with n = 3. *p < 0.05 as compared with TRPV1 by Student’s t.

(FIG. 3 B). However, the percentage of channel that reached the plasma membrane, measured as the ratio of surface/total TRPV1 protein, remained similar for all channel species (Fig. 3 B). Similarly, the TRPV1-AD2 mutants exhibited an electrophoretic profile and expression level similar to those of the AD2-4 and AD2-17 chimeras (data not shown). Thus, these results suggest that the chimeras were synthesized and trafficked to the plasma membrane, and imply that the complete lack of channel activity of the nonresponding chimeras may not be attributed to the absence of protein at the cell surface.

Intriguingly, at variance with wild-type channels that showed a double protein band (TRPV1 and AD2-20), all of the chimeras, except for AD2-19, appeared as a single protein band lacking the upper band (Fig. 3 A). Since the higher-molecular-weight protein band corresponds to N-type glycosylated subunits (34), this observation suggests that most of the chimeric channels were not N-glycosylated. This result implies that chimeras may have an altered protein fold that prevents this translational modification. Nonetheless, we cannot fully discard the possibility of a level of N-glycosylation that is below the sensitivity of the immunoblot detection limit. Taken together, our findings suggest that the lack of function of the chimeras may be due to a folding defect that reduces protein expression at the plasma membrane and abrogates the response to the activating stimuli.

Functional chimeras require high depolarizing voltages for activation

To examine the role of mutated amino acids in polymodal gating, we characterized the biophysical properties of the channel gating of the functional chimeras AD2-18 and AD2-19, as well as the recovered wild-type channel (AD2-20). First, we investigated voltage-evoked channel activation. Fig. 4 A shows typical voltage-activated currents for TRPV1, AD2-20, mock-transfected cells, and chimeras AD2-18 and AD2-19. All other chimeras (AD2-1 to AD2-17) did not respond to 100 ms depolarizing potentials up to 300 mV (data not shown). The application of depolarizing pulses up to 240 mV evoked measurable noninactivating ionic currents from these chimeric species, although they exhibited significantly reduced activity as compared with the TRPV1 channels.

The functional impact of mutations present in chimeras AD2-18 and AD2-19 on voltage gating was determined from G-V curves (Fig. 4 B). Because voltage is a partial TRPV1 activator (6,22), we normalized the G-V curves with respect to the maximal channel conductance (Gmax) obtained in the presence of 10 μM and 100 μM of capsaicin for the wild-type and chimeras, respectively. Fitting the G-V curves to a Boltzmann relationship yielded a G50 = 135 ± 5 mV, zg = 0.65 ± 0.08, (G/ Gmax)max = 0.71 ± 0.07, and ΔG o = 1.92 ± 0.01 kcal/mol for TRPV1, and V0.5 = 146 ± 8 mV, zg = 0.68 ± 0.10, normalized (Gmax)max = 0.71 ± 0.07, and ΔG o = 1.93 ± 0.02 kcal/mol for AD2-20 (Fig. 4, B and C). Conversely, the partially activated mutants AD2-18 and AD2-19 displayed G-V relationships that were displaced toward higher depolarized membrane potentials. Fits to the Boltzmann equation of these chimeras should be taken cautiously since the experimental points are still very far from the maximal normalized conductance (G/Gmax ≤ 0.5), and therefore biophysical conclusions cannot be obtained. Nonetheless, for comparison purposes, the inferred values were V0.5 ≥ 250, zg ≈ 0.7, (Gmax)max ≈ 0.2, and ΔG o ≥ 4.0 kcal/mol for AD2-18, and V0.5 = 250 ± 24 mV, zg = 0.7 ± 0.2, (Gmax)max = 0.41 ± 0.09, and ΔG o = 4.0 ± 0.6 kcal/mol for AD2-19 (Fig. 4, B and C). Thus, these two chimeric mutants showed significantly larger V0.5 and ΔG o values than TRPV1 but maintained a similar zg value. These results indicate that conservative mutations present in AD2-18 and AD2-19 are pivotal for the correct voltage-dependent activation of TRPV1 channels.

Functional chimeras display voltage-dependent capsaicin responses

We also investigated the response of functional chimeras to capsaicin at both negative and positive membrane potentials...
We evaluated the current-to-voltage (I-V) relationship from −80 to 160 mV in the presence of 1 μM or 100 μM of capsaicin (Fig. 5, A and B). The reinstated wild-type (AD2-20) responded to both capsaicin concentrations to an extent similar to that observed for the TRPV1 channels. AD2-19 exhibited smaller current values than TRPV1 in the presence of 1 μM of capsaicin, and AD2-18 showed lower responses than the wild-type at both vanilloid concentrations. Chimeras AD2-18 and AD2-19 exhibited strong inward rectification at negative membrane potentials (Fig. 5, A and B).

To evaluate the capsaicin responses of these channels, we examined the G-V curves at both agonist concentrations (Fig. 5, C and D). TRPV1 and AD2-20 transfected cells exposed to 1 μM of capsaicin displayed G-V curves with a significant voltage-independent component \( G_{\text{min}}/G_{\text{max}} \) at negative membrane potentials \( (0.36 \pm 0.07 \text{ for TRPV1 and } 0.4 \pm 0.1 \text{ for AD2-20}) \), along with a voltage-dependent phase at positive potentials (Fig. 5 C). In contrast, AD2-18 and AD2-19 chimeras exhibited only the voltage-dependent phase in the presence of 1 μM of capsaicin. An increment of the vanilloid concentration to saturation (100 μM) fully activated TRPV1 and AD2-20 channels in a voltage-independent manner (Fig. 5 D). This high concentration moved the G-V curves of AD2-18 and AD2-19 chimeras leftward toward less depolarized potentials (Fig. 5 E). This vanilloid concentration also evoked detectable voltage-independent gating for AD2-19 and \( G_{\text{min}}/G_{\text{max}} = 0.33 \pm 0.09 \), but not for the AD2-18 channel. These results imply that both chimeric channels displayed a reduced sensitivity to the vanilloid, since their voltage-independent responses were significantly lower than those of the TRPV1 channels.

An analysis of the voltage-dependent component substantiates the impaired response of chimeras AD2-18 and AD2-19 to changes in membrane potential, since they displayed higher \( V_{\text{0.5}} \) and \( \Delta G_o \) values than the TRPV1 channels (Fig. 5, E and F). However, the presence of the vanilloid did not significantly alter the gating valence \( (z_g = 0.5\text{–}0.6e) \) of the voltage-dependent component.

**Functional chimeras require higher activation temperatures than TRPV1**

To obtain the thermodynamics of channel activation, we studied the temperature-dependent gating of chimeric mutants. For this purpose, we performed direct, time-resolved measurements of heat activation by using fast temperature jumps and patch clamp (5,28). The temperature was changed from 36° to 60°C for AD2-18 and AD2-19, and from 36° to 54°C for the wild-type in 100 ms, and currents were recorded at −60 mV and +60 mV. Fig. 6 depicts the responses for TRPV1 and representative AD2 chimeric mutants evoked by the temperature steps at both voltages. Chimeras TRPV1-AD2, AD2-4, and AD2-14 were completely insensitive to the range of heating temperatures applied at both potentials (Fig. 6 A) and only showed noisy leak currents, which were likely due to membrane thermal alteration and activation of endogenous channels. In contrast, chimeras AD2-18 and AD2-19 and the reinstated wild-type (AD2-20) were activated by heat (Fig. 6 B). At −60 mV, chimeras AD2-18 and AD2-19 started to respond at 54°C and 51°C, respectively, whereas the wild-type channels (AD2-20) activated at 42°C, in accordance with previous results (5). At depolarized potentials, a decrease in the...
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channel activation (≤ 36°C) and the temperature sensitivity (Q10 ≈ 3.0) for both TRPV1 channels (Table 1). At depolarized potentials, the enthalpy of activation (ΔH = 32 kcal/mol for AD2-19 and 47 kcal/mol for AD2-20) was reduced without altering the T₀.₅ (56°C ± 4°C for AD2-19 and 48°C ± 2°C for AD2-20) (Table 1).

We further characterized the thermodynamics of activation for both channel species using the van’t Hoff relationship and assuming a two-state model. As illustrated in Fig. 7 C, the best linear fit of the experimental data gave a ΔH = 80 ± 5 kcal/mol and ΔS = 240 ± 28 cal mol/K for the AD2-19 chimera, which are significantly lower than the values obtained for AD2-20 (ΔH = 107 ± 5 kcal/mol and ΔS = 330 ± 43 cal mol/K). As a result, the free energy of activation for AD2-19 was larger than that of the wild-type (AD2-20) (Fig. 7 D; Table 1). Akin to what was found for the wild-type, the large enthalpy associated with channel activation was largely compensated for by the change in entropy, thus keeping the ΔG low enough to ensure fast activation. Notably, the activation energy (Ea) obtained from an Arrhenius plot displayed the same tendency, i.e., it was lower for AD2-19 (Ea = 34 kcal/mol) than for AD2-20 (Ea = 40 kcal/mol) (Fig. 7 E; Table 1). Despite these differences in the thermodynamics of channel activation by temperature, the enthalpy and entropy changes were coupled in both channels (Fig. 7 F). Taken together, these results indicate that chimera AD2-19 retained temperature-dependent gating, although with lower sensitivity and higher energetics than the wild-type channels.

The channel gating of chimera AD2-19 and the wild-type (AD2-20) can be described with an allosteric model

TRPV1 gating has been proposed to be an allosteric process whereby the activating sensors are coupled to the channel gate (6,7,35). Mutations in the TRP domain have been proposed to affect allosteric channel activation (22,36). Thus, we use a 16-state allosteric model for channel gating to evaluate whether it can describe the activation of AD2-19 and AD2-20 channels by the different activating stimuli (Fig. 8 A) (6). The model assumes the existence of independent sensors that are coupled to the channel gate and move simultaneously (6,7). According to this model, the equilibrium constants J and K govern the transitions between the resting (R₀) and active (A₀) states of the voltage and temperature sensors, respectively. The constant Q determines the equilibrium between the unbound (U) and bound (B) states of the capsaicin-binding site, and L drives the transitions between the closed (C) and open (O) states (Fig. 8 A). The voltage and temperature sensors and capsaicin-binding sites are coupled to pore opening by the allosteric constants D, M, and P, respectively, and to each other by E₁, E₂, and E₃. If we assume that all sensors are independent, then E₁ = E₂ = E₃ = 1.0.
In the absence of capsaicin, channel activation is mainly driven by membrane depolarization and temperature, i.e., the voltage and temperature sensors. Under this condition, considering the presence of four sensors for voltage and temperature in the receptor (one for each subunit), the probability of channel gating is given by (35,37)

\[
P_o(V, T) = \frac{1}{1 + \left(1 + J + K + JK\right)^4} \quad \text{where} \quad J = J_o e^{z_g V} \quad \text{and} \quad K = e^{(\Delta H - T \Delta S)/RT}.\]

(1)

To reduce the fitting complexity of Eq. 1, which has seven parameters, we kept some of them fixed and used both G-V and G-T curves to find the best values that described both relationships. We used the \(z_g\) value obtained from the Boltzmann fit (\(z_g = 0.65\)), and the \(\Delta H\) and \(\Delta S\) values obtained from the thermodynamic analysis (Table 1). The G-V data were fitted at 295°C (Fig. 8 B) and the G-T data were fitted at \(V = -60\) mV (Fig. 8 C). Under these conditions, the normalized G-V and G-T data for wild-type channels (AD2-20) were fairly described with the following values for the constants: \(J_o = 0.05 \pm 0.01\), \(L = 0.010 \pm 0.004\), \(D = 3.5 \pm 0.3\), and \(M = 10 \pm 1\) (\(R^2 = 0.98\) for G-V and 0.96 for G-T). For the AD2-19 chimera, the fit was worse because the data did not reach the maximal conductance. Since the TRP domain does not appear to be part of the sensors or the channel gate, we assumed that the AD2-19 chimera would exhibit \(J_o\) and \(L\) constants similar to those of the wild-type channels. With this assumption, we determined that the curves could be described with \(D = 2.1 \pm 0.5\) and \(M = 8.2 \pm 0.4\) (\(R^2 = 0.75\) for G-V and \(R^2 = 0.93\) for G-T). Therefore, although this analysis should be considered with caution because the analyzed data are based on macroscopic conductance rather than real open probabilities, it suggests that mutations in the TRP domain primarily affect the coupling constants of the voltage and temperature sensors to the channel gate.

To further corroborate this result, we also analyzed capsaicin-evoked gating in the wild-type (AD2-20) and the AD2-19 chimera at 295°C and \(V = -60\) mV using the allosteric model. For this analysis, we used the relationship (6,35,37)

\[
P_o(V, T, \text{Cap}) = \frac{1}{1 + \left(1 + J + K + Q + JK + QK + JQK\right)^4} \quad \text{where} \quad Q = \frac{[\text{capsaicin}]}{K_D},
\]

(2)
where $K_D$ is the capsaicin dissociation constant. To simplify the fitting, we used the $z_g$ value from the Boltzmann fitting (Fig. 5). $\Delta H$ and $\Delta S$ values from the van’t Hoff analysis (Table 1), $J_o$ and $L$ values from the analysis in the absence of agonist (Fig. 8, B and C), and $K_D = 10^{-5}$ $\mu$M for capsaicin (6,22). As depicted in Fig. 8 D, normalized $G$-$V$ curves in the presence of 1 $\mu$M of capsaicin were reasonably described by the allosteric model ($R^2 = 0.96$). Wild-type channels (AD2-20) exhibited a $P = 22 \pm 3$, whereas the chimera AD2-19 channel displayed a lower value ($P = 6.4 \pm 1.8$). Fitting these $G$-$V$ data to the allosteric model did not significantly alter the value of $D (3.3 \pm 0.4$ for AD2-20 and $2.6 \pm 0.2$ for AD2-19) or $M (9.6 \pm 0.3$ for AD2-20 and $7.9 \pm 0.5$ for AD2-19). Raising the capsaicin concentration to 100 $\mu$M gave $D = 2.7 \pm 0.3$, $M = 8.2 \pm 0.2$, and $P = 3.6 \pm 1.5$ for AD2-19 (data not shown). Taken together, these data suggest that mutations in the TRP domain impair channel gating by altering the allosteric coupling of the activating sensors to the channel gate, and imply that the TRP domain may be a determinant for polymodal gating in TRPV1 channels.

### DISCUSSION

The TRP domain has been implicated as a key structural element for allosteric activation of the channel pore (21–23). Furthermore, swapping this domain in TRPV1 with the cognate region from TRPV2 results in a nonfunctional TRPV1-AD2 chimera (21), despite the sequence similarities between both TRP domains. A recently reported structural model for TRPV1 portrays the TRP domain as an $\alpha$-helix that runs parallel to the inner leaflet of the lipid bilayer and interacts with several intracellular regions of the channel from the S4-S5 linker to the pre-S1 domain (26), thus implying that the complete TRP domain may be pivotal for channel function. To examine this hypothesis, we performed a step reconstruction of the TRP domain in the nonfunctional TRPV1-AD2 chimera to identify the minimum number of molecular determinants that led to the restoration of channel function.

The most salient contribution of this study is the finding that nearly the entire TRP domain appears to be needed to recover channel activity in the TRPV1-AD2 chimera. Indeed, our data indicate that correct channel gating occurred when the TRP domain contained the wild-type sequence. Even conserved mutations in highly conserved positions in this domain resulted in a decrease in channel gating. Channel responses to voltage, capsaicin, and temperature were progressively obtained when more than 90% of the TRP domain sequence of TRPV1 was reinstated in the chimeric TRPV1-AD2 channel. This strategy identified residues E692, K701, and S704 as molecular determinants for TRPV1 channel function. Conservative and cumulative mutation of these residues in TRPV1 (TRPV1(E692D/R701K/T704S)) gradually led to abrogation of channel function. This result suggests that mutation of these positions in TRPV1-AD2 may suffice to endow functionality to the chimera, regardless of the other residues in AD2. However, mutation of these residues alone in the nonfunctional
chimera (TRPV1-AD2(D692E/K701R/S704T)) did not produce functional channels, implying that other positions in the TRP domain are concomitantly required for restoring channel function. This inference is consistent with the impaired activity observed in the TRPV1-AD3 chimera, where the AD1 domain was replaced by the cognate from the TRPV3 channel (21). The TRPV1-AD3 chimera, which exhibits E692, R701, and T704 residues akin to TRPV1 wild-type channels, did not respond to voltage, responded barely to temperature activation, and displayed impaired capsaicin responses (21). Taken together, these findings suggest that the integrity of the TRP domain is important for correct TRPV1 gating.

Molecularly, we found that nonfunctional chimeras produced lower levels of protein than TRPV1, although the expressed protein was trafficked to the plasma membrane in a similar ratio as wild-type channels. In addition, we also observed that nonfunctional chimeras apparently lack the N-type glycosylation that is characteristic of TRPV1. Our results suggest that the recovery of channel function was paralleled by an increase in the level of protein expression, and the occurrence of N-glycosylation of the receptor subunit. Indeed, N-type glycosylation was detected in functional chimera AD2-19 and fully recovered in restored wild-type channels (AD2-20). It should be noted that the absence of N-type glycosylation cannot account for the channel unresponsiveness of TRPV1-AD2 chimeras because mutation of the N-glycosylation site or pharmacological abrogation of N-type glycosylation produces non-glycosylated TRPV1 channels that are fully functional (16,34). Thus, the lack of functionality of TRPV1-AD2 chimeras could be due to a lower protein expression at the plasma membrane and/or to a folding defect that impairs channel gating. However, the minor surface expression of chimeras cannot be the only cause of their lack of function. For instance, although the expression levels of TRPV1-AD4 and TRPV1-AD18 are similar, the former chimera is completely inactive. Thus, a plausible explanation for the lack of activity of most chimeric channels is that mutations of the TRP domain affect protein folding, which influences the allosteric activation of the channel. A contribution of the TRP domain to the tertiary and/or quaternary structure of the channel seems feasible, as suggested by 1) the effect of mutating this region on N-glycosylation of the receptor, 2) its contribution to subunit oligomerization (24), and 3) its putative structure in the protein, which depicts several inter- and intrasubunit contacts with intracellular domains encompassing the entire transmembrane domain (S1–S6) (26).

A biophysical analysis of channel gating revealed that mutations in the TRP domain raised the activation energy, requiring stronger activating stimuli for active chimeras (AD2-18 and AD2-19) than the wild-type, and unreachable levels for the nonfunctional channels (AD2-AD2-17). A comparison of AD2-19 and wild-type gating using an allosteric activation model suggested that mutations in the TRP domain mainly affected the allosteric coupling of the activating sensors and the channel gate, with minor effects in the function of the sensors. This finding implies that coupling the activating stimuli to the opening of the channel gate seems to be a highly sensitive step in channel gating that depends on the fine-tuning of a constellation of interactions at the level of the TRP domain, consistent with the reported
structure of this domain (26) and the conformational change induced by activating ligands (27). Furthermore, it substantiates that this protein region is important for conveying the conformational changes evoked by the activating stimuli to pore gating. The smallest alteration of the protein-protein interactions mediated by this domain apparently suffices to raise the energetics of the activation process.

Previous studies have shown a contribution of the TRP domain to ligand- and voltage-dependent gating (22), and suggested that temperature-induced gating was also affected (21,23). Here, we additionally studied the contribution of this protein region to temperature-mediated activation and the thermodynamics of channel gating. Our data imply that the TRP domain is also pivotal for linking the conformational changes in the temperature sensor to the opening of the gate. Mutation of the TRP domain mainly affected the channel sensitivity to temperature, reducing the $Q_{10}$ and increasing the threshold and $T_{0.5}$ for gating. As a result, a decrease in the enthalpy and entropy associated with the gating process was observed in chimeric AD2-19 channels, which resulted in an augment of $\Delta G$ for these channels.

Our data suggest that mutations of the TRP domain uncouple the temperature sensor and the channel gate. However, the existence of a specific temperature sensor in the thermoTRP channel family has been questioned (4,9,35,38), and an alternative, three-tiered allosteric model for heat-induced gating was recently proposed for TRPV1 and Kv channels (39) and for cold-induced activation of TRPM8 (37). According to this model, the gating mechanism can be represented in a simplified way as $C \leftrightarrow C' \leftrightarrow O$, where the $C' \leftrightarrow O$ equilibrium is mainly driven by temperature and $C \leftrightarrow C'$ is a voltage-dependent transition. This scheme is consistent with the fast kinetics of temperature-induced gating, the lower threshold of activation, and the smaller $Q_{10}$ value of channel gating at depolarized potentials, as well as with the lack of voltage dependency of the $T_{0.5}$ value, since the $C' \leftrightarrow O$ transition is essentially voltage independent. In addition, it provides support for the role of the TRP domain in channel gating, as mutations in this region essentially modulate the energetics of the $C' \leftrightarrow O$ transition. However, fitting the $G-T$ data to an allosteric model reveals that whereas mutations of this protein domain only marginally affect the equilibrium constant of the $C' \leftrightarrow O$ transition, they do uncouple it from heat sensing. Although this conclusion is derived from the allosteric model used here, which assumes the existence of an independent temperature sensor, it is also compatible with the TRP domain playing a role in transmitting the temperature-induced conformational change to the channel pore without altering the gating equilibrium constant, which will mainly depend on protein-protein interactions at the level of the gate. Nonetheless, additional experimental support is needed to understand the precise biophysical and molecular mechanisms underlying the polymodal activation of TRPV1, and to discern whether a temperature sensor exists within the channel structure.

Recent structural models for TRPV1 at 3.4 Å in closed and open conformations, derived from cryo-EM images, provide pivotal molecular blueprints for understanding the necessity of a complete TRP domain for efficient gating (26). The proposed structural arrangement of the TRP domain gives support to the importance of the conserved mutations that led to the rescue of channel function in chimeras AD2-18 (K701R) and AD2-19 (D692E) (Fig. 9). The TRPV1 cryo-EM structure reveals that R701 in

![FIGURE 9 Molecular models of the TRP domain of TRPV1-AD2 mutants. (A–F) Details of the models, showing the neighborhood of positions R701 (A and B), E692 (C and D), and T704 and D707 (E and F), and the changes in the interaction patterns observed after mutation.](image-url)
TRPV1 is hydrogen bonding with Q423 in the pre-S1 domain of an adjacent subunit, and may be forming an intra-subunit π-cation interaction with W697 in the TRP domain (Fig. 9A). In addition, this region may be involved in PI(4,5)P2 modulation (40). Incorporation of a Lys at this position disrupts these interactions, thus changing the conformation of the protein (Fig. 9B). Position E692 in TRPV1 appears close to H410 in the pre-S1 region and K688 (Fig. 9C). Mutation of D692E in the structural model appears to facilitate the formation of a hydrogen bond with N695 in the TRP domain, reorienting this residue away from H410 and K688 (Fig. 9D). Mutation of S704T, which virtually restores the wild-type folding, reveals a conformational change that is mainly observed as an outward turn at Y401 in the pre-S1 domain, which becomes more exposed (Fig. 9, E and F). This structural change is also facilitated by having an Asp at position 707. Therefore, considering that agonist-induced channel opening is produced by subtle structural changes at the TRP domain (27), mutations that change its conformation could be expected to have a significant impact on the channel functionality.

In conclusion, our findings indicate that the TRP domain in TRPV1 is a key structural and functional determinant that is essential for channel gating. Notably, the entire sequence of this protein region appears to be important for channel function. The constellation of intra- and intersubunit interactions of this domain, as revealed by the cryo-EM structure (26), may couple the conformational changes in the putative sensors for the activating stimuli to the opening of the aqueous pore. In support of this tenet, a recent study with TRPV4 concluded that interactions of the TRP box with the S4-S5 linker stabilize the closed state of the channel (41), suggesting that the TRP domain may directly couple the sensors with the channel gate. Thus, there are still important unanswered questions that require additional experimentation to unveil the specific mechanistic details of gating in this polymodal receptor, as well as its regulation by PI(4,5)P2. In this regard, the library of TRP domain mutants, along with analysis of their single-channel activity, may pave the way for understanding the mechanism of TRPV1 allosteric gating and to unveil the role of the TRP domain. Our results also corroborate the notion that the interactions of the TRP domain with cytosolic protein domains may aid in the development of allosteric modulators of channel gating (25). Because the TRP domain is present in several members of the TRP channel superfamily, these allosteric modulators may increase the pharmacological armamentarium for modulation of TRP channels.

AUTHOR CONTRIBUTIONS

L.G.-T. performed all of the mutagenesis, Ca2+ microfluorography, and immunoblots, and participated in the characterization of functional activity. P.V. performed the electrophysiological characterization of chimeras. B.L. contributed to the characterization of the thermal activation of channel species. G.F.-B. performed the molecular modeling. F.Q. contributed to the design of the thermal activated experiments and supervised data processing. A.F.-M. designed the experiments, analyzed the data, and wrote the manuscript.

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