THE TRP BOX OF THE VANILLOID RECEPTOR IS A MOLECULAR DETERMINANT OF FUNCTIONAL COUPLING

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TRPV1 (transient receptor potential vanilloid receptor subtype I) is an ion channel gated by physical and chemical stimuli that belongs to the TRPV protein family. TRPV receptors contain a highly conserved, 6-mer segment near the channel gate, known as the TRP box, whose role in channel function remains elusive. Here, we performed an Alanine scanning mutagenesis of the TRP box of TRPV1 (IWKLQQR) and found that mutation of this motif affected channel gating by raising the free energy of channel activation. Functional characterization of TRPV1 mutants showed that substitution of I696, W697 and R701 by A severely compromised voltage- and heat-dependent activation and notably reduced the capsaicin responsiveness, while mutation of K698, L699 and Q700 had minor effects. In addition, mutation of I696 to A promoted strong outward rectification at negative membrane potentials, and significantly slowed the kinetics of channel activation. Collectively, our findings suggest that modification of I696, W697 and R701 altered channel gating by affecting events downstream of the initial stimuli sensing step, and imply that intersubunit interactions within the TRP box contribute to functional coupling in TRPV1.

The capsaicin receptor is a member of the TRP ion channel superfamily that gave name to the vanilloid subfamily (TRPV) of receptors (1;2). This membrane protein plays an important role in noxious sensing and pain perception. TRPV1 is activated by harmful temperatures (≥42°C), and exhibits remarkable heat sensitivity, with a Q10 ≥20 (3;4). A seminal study concluded that the channel thermosensitivity of TRPV1 arises from the difference in activation energies associated with voltage-dependent gating (5;6). In addition, this channel protein is gated by vanilloid molecules like capsaicin and resiniferatoxin, extracellular pH, and pro-inflammatory substances (1;2;7;8). It has been proposed that chemical agonists of this thermoreceptor function as gating modifiers that mimic and potentiate the thermal responses (5).

A functional TRPV1 channel is a homotetramer of subunits assembled around a central aqueous pore (9;10). The overall topological organization of TRPV1 subunits is akin to that displayed by the hexahelical Shaker-like K⁺ channels, having six transmembrane segments (S1-S6), and cytosolic N- and C-termini (2). Whereas the N-terminus contains ankyrin domains that contribute to channel assembly and function (11-16), the C-terminus appears important for subunit tetramerization, temperature sensing and modulation of channel gating (17-20). Near the channel gate, there is a 6-mer segment, referred to as the TRP box, that is highly conserved among the TRP channel family (2). In TRPM channels, this motif is implicated in the modulation of channel activity by phosphoinositides (21-
23), as well as it seems to mediate the coupling of menthol binding to channel opening (24). These observations strongly suggest that the TRP box may also serve as a molecular determinant of TRPV1 channel function.

By using an Alanine scanning mutagenesis approach of the entire TRP box, we found that this motif plays a role in functional coupling and identify I696, W697 and R701 as molecular determinants of channel gating. We report that replacement of these residues by A notably impaired capsaicin, voltage and heat-induced channel activity by a mechanism that is consistent with the uncoupling of stimuli sensing from pore opening. These findings support the notion that inter- and/or intra-subunit interactions at the level of the TRP box in TRPV1 contribute to define the activation energy of channel opening.

**Experimental procedures**

**TRPV1 receptor mutagenesis.** Site-directed mutagenesis of the residues encompassing the TRP box to Alanine was carried out by PCR as described (25). Mutant receptors were confirmed by DNA sequencing. For mutants, the number indicates the position of the residue in the protein sequence; the first letter is the natural amino acid in the wild type protein and the second is the residue that substitutes it.

**Cell culture and transfection.** HEK293 cells were cultured in DMEM supplemented with 10% foetal calf serum, 2 mM L-glutamine and 1% penicillin-streptomycin solution at 37°C in 5% CO₂. Cells were transfected with 2 µg of DNA encoding the TRPV1 and mutant channels with Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s recommendations. Cells were used 48 h after transfection.

**Intracellular Ca²⁺ imaging.** Cells were plated at a density of 10⁶ cells/cm² onto poly-D-lysine-coated 25 mm coverslips. Cells were transfected with 2 µg of DNA encoding the TRPV1 and mutant channels with Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s recommendations. Ca²⁺ image measurements were carried out 48 h after transfection. Cells were incubated with 5 µM Fluo-4 AM (Molecular Probes) in the presence of 0.02% pluronic F-127 (Biotium) in isotonic standard solution (in mM: 140 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 5 glucose, 10 HEPES pH 7.4; the osmolarity was adjusted to ≈315 mOsm with manitol (Gonotec Osmometer)) for 1h at 37°C. For Ca²⁺ imaging, cells were continuously perfused (1 ml/min) with isotonic standard solution at ≈22°C. TRPV1 activity was evoked with 10s pulses of capsaicin (1 and 100 µM) using a multibarreled, gravity-driven perfusion system. Fluorescence measurements were carried out with a Zeiss Axiovert 200 inverted microscope fitted with an ORCA-ER CCD camera (Hamamatsu) through a x20 water immersion objective. Fluo-4 was excited at 500 nm using computer controlled Lambda 10-2 filter wheel (Sutter Instruments), and emitted fluorescence filtered with a 510 nm long-pass filter. Images were acquired and processed with AquaCosmos package software.

**Patch clamp measurements in HEK293 cells.** HEK293 cells were co-transfected with TRPV1 species and the EYFP protein (pEYFP, Clontech). Membrane currents were recorded with the whole cell configuration using patch clamp as described (26). For whole cell recordings, pipette solution contained (in mM): 150 NaCl, 3 MgCl₂, 5 EGTA and 10 HEPES, pH 7.2 with CsOH, and bath solution contained (in mM): 150 NaCl, 6 CsCl, 1 MgCl₂, 1.5 CaCl₂, 10 glucose and 10 HEPES, pH 7.4 with NaOH. The different saline solutions were applied with a gravity-driven local microperfusion system with a rate flow of ~200 µl/min positioned at ~100 µm of the recorded cells. All measurements were performed at 20-22°C. A voltage step protocol consisting of 50 ms depolarizing pulses from -120 mV to 300 mV in steps of 20 mV was used. The holding potential was 0 mV and the time interval between each pulse was 5s. I-V relationships were studied using a ramp protocol consisting of a voltage step of 300 ms from the holding potential of 0
mV to -120 mV, followed by 350 ms linear ramp up to 160 mV. Time interval between each pulse was 10 s. 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 less than 10 MΩ and to minimize voltage errors was compensated to 70-90%.

The G-V curves were obtained by converting the maximal current values from a 100 ms voltage step protocol (from -120 to +160 mV) to conductance using the relation \( G = \frac{I}{(V-V_R)} \), where \( G \) is the conductance, \( I \) is the peak current, \( V \) is the command pulse potential, and \( V_R \) is the reversal potential of the ionic current obtained from the I-V curves. Conductance values from different cells were normalized to that of +160 mV, and fitted to the Boltzmann equation: \( G = \frac{G_{\text{max}}}{1 + \exp \left( \frac{(V-V_{0.5})/a_n}{n} \right)} \), where \( G_{\text{max}} \) is the maximal conductance, \( V_{0.5} \) is the voltage required to activate the half-maximal conductance and, \( a_n \) is the slope of the G-V curve. The free energy difference between the closed and the open states at 0 mV and 25ºC for a two-state model (\( \Delta G_0 \)) was calculated from \( \Delta G_0 = z_g F V_{0.5} \) (27), where \( F \) is the Faraday constant (0.023 Kcal/mol mV), and \( z_g \) is the apparent gating valence obtained using \( z_g = 25.69 \text{ mV/a}_n \). Data were analysed with PulseFit 8.11 (HEKA Elektronik), and Origin 7.0 (MicroCal). Data were expressed as mean±SD, with \( n = \) number of cells. Statistical analysis was performed using the 1-way ANOVA, and the \( p < 0.05 \) was taken as the level of significance.

Results

Mutation of the TRP box altered TRPV1 capsaicin responsiveness. The TRP box is a highly conserved protein motif located at the core of the TRP domain in TRPV channels (19), as well as in other TRP proteins (2). To investigate its functional role, we performed an Alanine scanning mutagenesis of all residues encompassing this region in TRPV1 (Fig. 1A), and characterized the phenotype of mutant channels in HEK293 cells. Mutation of TRP box residues did not alter subunit multimerization and channel trafficking to the plasma membrane as implied by the co-localization of mutant proteins with the cell surface marker wheat agglutinin (Supplemental information (SI), Fig. S1). All mutant subunits gave rise to functional channels that exhibited capsaicin-evoked Ca\(^{2+}\) influx (Fig. 1B), but displayed distinct sensitivities to the vanilloid. Whereas mutation of I696, W697 and R701 to A produced proteins that did not respond 1 \( \mu \)M capsaicin, mutants K698A, L699A and Q700A were activated by this low agonist concentration (Fig. 1C, top). All mutants, however, responded to 100 \( \mu \)M capsaicin (Fig. 1C, bottom). Notice that mutant I696A exhibited a limited Ca\(^{2+}\) entry at the high vanilloid concentration, as evidenced by the fast recovery of the baseline level upon termination of the vanilloid pulse.
These observations indicate that mutants I696A, W697A and R701A display lower capsaicin sensitivity than K698A, L699A and Q700A mutants. Results obtained from *Xenopus* oocytes heterologously expressing K698A, L699A and Q700A mutants corroborated that they produce capsaicin responses akin to those of TRPV1, and substantiated that I696A, W697A and R701A mutants were poorly activated by the vanilloid (data not shown). Thus, residues I696, W697 and R701 appear to be more sensitive to replacement than K698, L699 and Q700.

We next investigated the vanilloid sensitivity of I696A, W697A and R701A mutants expressed in HEK293 cells by obtaining their respective capsaicin dose-response relationships using patch clamp. As illustrated in Fig. 2A, the three mutants responded to capsaicin in a dose-dependent manner. Analysis of the vanilloid dose response curves revealed an EC50 of 3.6 ± 0.4 μM (n=4) for I696A, 6.3 ± 0.7 μM (n=4) for W697A and 43 ± 3 μM (n=4) for R701A (Fig. 2A), which is significantly higher than that of TRPV1 (0.6 ± 0.1 μM, n=4). For the three mutants, the Hill coefficient was significantly reduced (Fig. 2B), suggesting that subunit cooperativity during the gating step was also affected in these channels. Furthermore, their low capsaicin responsiveness was accompanied by a significant decrease in the maximal response evoked by the vanilloid (Fig. 2C). Taken together, these results demonstrate that mutation of I696, W697 and R701 to A reduced the capsaicin sensitivity. Furthermore, our data suggests that the change in the vanilloid EC50 of the mutants is contributed to a significant degree by the reduction in the maximal response evoked by capsaicin, compatible with the notion that mutation of these residues in the TRP box affected events downstream of the initial binding step such as channel gating.

**Mutation of the TRP box compromised TRPV1 voltage-dependent gating.** To further characterize the effects of mutating the TRP box on channel gating, we studied the voltage-dependent activation of mutant channels with whole-cell patch clamp (5). Stimulation with 50 ms depolarizing voltage steps from -120 mV to +300 mV elicited non-inactivating macroscopic currents from TRPV1 and I696A, K698A, L699A and Q700A mutants, but not from cells expressing the W697A (n=20) and R701A (n=22) channels or mock transfected cells (n=6) (Fig. 3A). All active mutants displayed a current density lower than that of wild type channels, being remarkably small (≤10% of TRPV1) that of the I696A mutant (Fig. 3B). Mutation of K698, L699 and Q700 to A did not affect the kinetics of voltage-dependent channel activation from 120 to 160 mV (SI, Fig. S2) nor the reversal potential of the channel (SI, Fig. S3). The small ionic currents exhibited by the I696A mutant precluded their kinetic analysis.

Conductance-to-voltage (G-V) curves yielded a V0.5 for TRPV1 of 158 ± 3 mV (n=8), and a gating valence (zg) of 0.75 ± 0.07 (Figs. 4A-C), in accordance with previous values (5;18). The K698A mutant exhibited a ~20 mV (186 ± 3 mV, n=7) rightward change in the V0.5, whereas mutation of L699 and Q700 to A shifted the voltage-dependent activation to 210 ± 2 mV (n=8) and 216 ± 3 mV (n=7), respectively (Figs. 4A and B). Notably, replacement of I696 with A moved the V0.5 to values ≥300 mV (n=10) (Fig. 4B). This value is probably an underestimation because we could not reach saturation of the conductance curve with the voltage protocol used (Fig. 4A). None of these mutations appear to alter the apparent gating valence moved by the change in voltage (Fig. 4C), a measure of the voltage sensitivity of the activation process (28). Therefore, these results suggest that mutation of the TRP box altered the voltage-dependent gating presumably by raising the free energy of voltage activation. This notion was substantiated upon evaluation of the free energy difference between the closed and the open states at 0 mV and 25ºC (ΔGc) calculated from the values of V0.5 and zg considering a two state model (27). Although TRPV1 gating may be more complex than a two state model (5;29;30), this approach is a simple and valuable means to compare the effect of mutations on the energetics of channel gating. In addition, it has been shown that a two-state model appropriately
describes the voltage dependent gating of the structurally related TRPM8 channel (30). Fig. 4D depicts the values of $\Delta G_\text{o}$ obtained for TRPV1 and mutant channels. For TRPV1, the $\Delta G_\text{o}$ was 2.1±0.3 kcal/mol. Inspection of the plot reveals that K698A displayed a $\Delta G_\text{o}$ similar to wild type, whereas L699A and Q700A augmented $\Delta G_\text{o}$ by ≈0.7 Kcal/mol. Noteworthy, replacement of I696 with A increased $\Delta G_\text{o}$ by a significant 1.8 Kcal/mol. Therefore, these results imply that mutation of residues encompassing the TRP box has an impact on the activation energy of voltage-dependent gating, and signal to I696, W697 and R701 as important molecular determinants of channel opening.

**Mutant channels I696A, W697A and R701A exhibit voltage-dependent gating in the presence of capsaicin.** The impaired voltage-dependent activation of I696A, W697A and R701A could be due to abrogation of stimuli sensing or an alteration in channel gating that sets the activation energy at very high values. Since capsaicin displaces the G-V relationship of TRPV1 towards physiological voltages (5), we next evaluated its effect on the voltage activation of these three mutants and obtained their G-V curves in the presence of increasing concentrations of capsaicin. These experiments showed that: i) the three mutants exhibited voltage-dependent gating (Figs. 5A-C) and, ii) the vanilloid modulated differently their voltage-activated responses (Figs. 5D-F). A capsaicin-dependent, voltage-independent ionic conductance at negative membrane potentials was characteristic of W697A and R701A. In contrast, I696A exhibited a strong outward rectifying I-V relationship with barely detectable ionic current at hyperpolarized membrane potentials.

The agonist shifted the $V_{0.5}$ of I696A and W697A towards lower values in a dose-dependent manner, but it did not alter that of R701A (Fig. 5G). The apparent gating valence was unaffected by the presence of the vanilloid and was not significantly changed by the mutations (Fig. 5H). Analysis of the free energy of gating showed that capsaicin dose dependently reduced the free energy difference between the closed and the open states at 0 mV and 25°C of I696A and W697A mutants (Fig. 5J). R701 did not display a capsaicin-dependent change of the free energy of activation by voltage. When compared with wild type channels, mutants exhibited higher activation energies in the presence of a concentration of capsaicin that equals their respective EC$_{50}$ for the vanilloid, compatible with their higher energetics of channel gating (Fig. 6A). Taken together, these data suggest a contribution of residues I696, W697 and R701 to the activation energy of channel gating.

**Mutation of I696 to A stabilized the closed state of the channel.** To learn more on the I696A functional phenotype, we first examined the I-V relationship in the presence of increasing concentrations of the vanilloid (Fig. 5A). As depicted, the I696A mutant exhibited a distinct I-V characterized by a strong outward rectification to the entire range of the vanilloid concentrations tested. At variance with the W697A and R701 A (Figs. 5B and C), the I696A mutant did not show ionic current at negative potentials even in the presence of 100 μM of capsaicin. As a result, the estimated rectification index for this mutant significantly increased as a function of the vanilloid concentration (Fig. 6B). This outward rectification was not due to Ca$^{2+}$ nor Mg$^{2+}$ blockade of the mutant channel because the rectification index was unaffected by removal of both divalent cations from the external medium (data not shown), nor it appeared due to a change in its permeation properties since it displayed a reversal potential within the range of the other channels (SI, Fig. S4). Thus, the strong rectification suggests an impaired ionic permeation at negative potentials that could be, at least in part, to stabilization of the channel closed state under hyperpolarization. In support of this notion, this mutant exhibited a much slower kinetics of its voltage-dependent activation in the presence of capsaicin than TRPV1 and W697A and R701A mutants (Fig. 6C; and SI Fig. S4). The time constant for channel activation of I696A was accelerated by the vanilloid in a dose-dependent manner, although even at 100 μM capsaicin, it was 10-fold slower than
that of TRPV1 (Fig. 6C). Collectively, these findings imply that incorporation of a smaller hydrophobic amino acid at position I696 increases the stability of the closed state of the channel that requires higher energetic input for gating. A stabilization of the closed state for I696A is also consistent with the fast recovery of the Ca\(^{2+}\) transient evoked by 100 \(\mu\)M capsaicin (Fig. 1C) which plausibly arises from fast pore closing.

**Mutation of the TRP box altered the temperature sensitivity of TRPV1.** The C-terminus of TRPV1 is a key molecular determinant of heat sensing (18,23), we next investigated the heat sensitivity of our mutant channels to determine the contribution of the TRP box. For this purpose, we obtained temperature-evoked responses at -60 mV and +50 mV (SI, Fig. S5). Heat ramps activated ionic currents from K698A, L699A and Q700A mutants but not from I696A and W697A (SI, Fig. S5 and Fig. 7A). For the R701A mutants a barely detectable ionic current could be observed at high depolarized potentials (SI, Fig. S5 and Fig. 7A). Heat-evoked ionic currents from active mutants were similar to TRPV1, although their magnitude was smaller, especially at hyperpolarized membrane potentials (Fig. 7A). We next evaluated the voltage dependency of heat-activated currents by obtaining the I-V relationships for all channels at 45ºC (Fig. 7B). Mutants K698A, L699A and Q700A displayed I-V curves similar to TRPV1. Notably, the R701A mutant, that was insensitive to voltage changes at 25ºC, was voltage-gated at 45ºC (Fig. 7B). In marked contrast, the I696A and W697A mutants did not produce measurable heat-activated currents within the voltage range applied (up to +160 mV) (Fig. 7B). Thus, mutation of the TRP box affected the heat responsiveness of TRPV1. These findings point to I696 and W697 as two important determinants of heat sensing in TRPV1.

The lower temperature sensitivity of the mutants may arise from an alteration of the heat sensor or to a defect in the temperature-induced gating. To address if mutations affected heat sensing, we determined the threshold temperature of channel activation (defined as the temperature that activated 10% of the maximal response) from the I-T relationships of TRPV1 and active mutants at +50 mV. As illustrated in Fig. 7C, wild type and mutant channels displayed a similar threshold temperature of \(\approx36^\circ\)C (35-38ºC) for channel activation. Even for mutant R701A, that exhibits lower heat sensitivity than the other mutants, the threshold temperature was also \(\approx35^\circ\)C.

In contrast, mutant channels depicted an altered temperature-dependent gating as determined from the Q\(_{10}\) value obtained between 35º and 45ºC (6,29,31). Whereas TRPV1 showed a mean Q\(_{10}\) of 23±5 (n=8), mutant channels K698A, L699A and Q700A displayed a \(\approx50\%\) reduction in this parameter (Fig. 7D). For R701A, the Q\(_{10}\) value could not be properly calculated because of its small heat-activated currents. These data support the tenet that the lower heat responsiveness of TRP box mutants is probably due to an alteration of the temperature-induced channel gating, rather than a modification of the temperature sensor.

**Mutant I696A did not show temperature sensitivity.** The important effect of mutating I696, W697 and R701 to A on heat sensitivity suggests a critical role of these residues in coupling heat sensing to pore opening. To further address this issue, we investigated the effect of raising the temperature to 45ºC on the capsaicin response of these mutants. To investigate the heat potentiation, we used a capsaicin concentration (1 \(\mu\)M) that barely activated the mutants at 25ºC (Fig. 8). At variance with the I696A mutant (Fig. 8A), the heat and vanilloid-evoked ionic currents of W697A and R701A were significantly potentiated by the simultaneous presence of both activating stimuli (Fig. 8 B and C), although W697A displayed a poorer heat potentiation than R701A. In contrast, for the I696A mutant the capsaicin responsiveness was unchanged by the rise in temperature (Fig. 8A). These data imply that both W697A and R701A preserve heat sensitivity and further substantiates that I696A has a severely compromised temperature-dependent gating that makes this channel mutant heat insensitive.
Discussion

The TRP box is a highly conserved motif in the cytosolic C-terminus domain of TRP channels (Fig. 9A). In TRPV1, this segment is at the core of the TRP domain, a coiled-coil structure that has a dual functionality serving as a molecular determinant of subunit multimerization and as a transduction domain important for efficient channel gating (19). Furthermore, the TRP domain of TRPM channels appeared to be required for phosphoinositide-mediated channel gating (22;32), as well as for activation with menthol (24); and for efficient transient light response in flies (33). Therefore, these observations suggest that the TRP box of TRPV1 may be functionally relevant. The salient contribution of our study is the demonstration that this protein motif in TRPV1 participates in channel gating by contributing to set activation energy of pore opening. By using an Alanine-scanning mutagenesis approach, we report that structural changes in the TRP box notably affected the response of the channel to capsaicin, voltage and heat. This effect was not due to loss of stimulus recognition since we found that mutation of TRP box residues did not change the ability of the channel to sense the gating stimuli, but it rather affected the gating reaction, plausibly by altering steps downstream of the initial sensing event. Our findings are compatible with a model in which residues of TRP box contribute to couple the activating stimuli to pore opening in TRPV1. Alteration or loss of specific inter- and/or intrasubunit interactions at the level of this motif has an important impact on functional coupling.

We found a differential contribution of TRP box residues to channel gating. Substitutions of K698, L699 and Q700 by A modestly affected channel function, and moderately augmented the free energy of channel opening. In marked contrast, mutation of I696, W697 and R701 drastically altered the sensitivity to the activating stimuli and raised the energetic barrier between the closed and open states of the channel. Replacement of I696, W697 and R701 with A significantly decreased the capsaicin responsiveness of the mutant channels. This effect on the capsaicin efficacy could arise from a change of the ligand affinity or to a modification of the activation step (34). The significant decrease in the maximal response elicited by the vanilloid in these mutants, along with their lower Hill coefficient, an indicator of the subunit cooperativity during channel gating (34), suggests that the lower capsaicin responsiveness arises primarily from an alteration of the gating step rather than the initial agonist binding event. Thus, this observation implies that the unliganded state of these mutants is similar to that of TRPV1 in their ability to undergo the allosteric, agonist-induced conformational change required for channel opening. Nonetheless, a change in agonist affinity produced by the mutations can not be completely ruled out, specially taking into account an allosteric protein such as TRPV1, since the ligand affinity depends on both the occupancy of the binding sites and the conformational state of the protein (34). Further experimental support is needed to address this issue.

Mutation of I696, W697 and R701 severely affected the ability of voltage to activate ionic currents in resting conditions, although they displayed voltage-dependent gating in the presence of capsaicin. The vanilloid shifted leftward the voltage-dependent channel activation towards lower potentials in a dose dependent fashion for I696A and W697A, but not as much for the less sensitive mutant R701A. This effect on the voltage dependency of channel activation was not accompanied by a change in the apparent gating valence. These observations imply that the distorted gating mechanism of I696A, W697A and R701A, which aroused primarily from attenuation of stimulus efficacy, is compatible with the notion that the mutations influenced events downstream of voltage sensing that lead to channel opening, plausibly they affected the coupling between stimulus sensing and channel gating. In addition, mutation of TRP box residues influenced heat sensitivity by decreasing the temperature-dependent gating as indicated by the lower Q10 value. Therefore, our findings indicate
that TRP box plays an important function in channel gating, and identify I696, W697 and R701 as important molecular determinants of functional coupling.

The TRP box of TRPV1 is located few amino acids after the putative channel gate at the core of the predicted coiled coil structured by the TRP domain (19), a position highly compatible with its role on functional coupling. Inspection of the TRP box sequence shows that amino acids I696 and W697 are located at the “a” and “b” positions of the putative coiled coil motif, where they could mediate intersubunit interactions of the four helix bundle assembled by TRPV1 subunits, as suggested by our previous model (Fig. 9B).

Substitution of I696 and/or W697 with a smaller amino acid could draw near the channel monomers, strengthening the subunit-subunit contacts near the channel gate, which would raise the coupling free energy for stimulus-mediated channel activation. Because of the central position of I696, the model predicts a stronger effect of mutating this residue than altering W697. Notice that the higher energy would arise at least in part from stabilization of the closed state of the channel. The remarkable high activation energy between the closed and open states exhibited by the I696A mutant, in conjunction with its slower activation time constant, lend support to such model. Furthermore, the strong outward rectification seen at negative potentials is compatible with an increase in the stability of the closed state.

In conclusion, our findings suggest that the TRP box of TRPV1 is a domain that may act as a coiled-coil zipper that contributes to hold the gate in the closed state, and imply that this motif could operate as a negative allosteric modulator of channel gating. The energy released by the activating stimuli could remove this inhibition by breaking intersubunit interactions that held the subunits together near the channel gate, favoring its opening.

The functional effect of mutating R701 to A is more intriguing since this amino acid does not seem involved in protein-protein interactions within the C-end of TRPV1 as residues I696 or W697 (Fig. 9B). This residue is one of the most conserved within the TRP box motives of TRPV, TRPM and TRPC (Fig. 9A). Noteworthy, mutation of the corresponding residue (R595) in TRPM8 produced a similar phenotype characterized by a lower menthol responsiveness and decreased sensitivity to PIP$_2$ modulation (24). Furthermore, a chimaeric TRPM8 channel incorporating the TRP domain of TRPV1 identified R701 as a molecular determinant of PIP$_2$ activation but not of menthol gating (23). Although some structural differences between TRPV1 and TRPM8 plausibly exist, collectively these findings lend support to a model where R701 would be oriented externally (Fig. 9B) (23), presumably interacting with the S4-S5 and PIP$_2$, which is also consistent with a role of this residue in coupling the activating stimuli to gate opening.
REFERENCES


**FOOTNOTES**

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**Abbreviations:** TRP, transient receptor potential; TRPV1 transient receptor potential vanilloid 1; TRPM, transient receptor potential melastatin subfamily; PIP2, phosphatidylinositol (4,5)-bisphosphate; Supplemental information, SI.

**FIGURES LEGENDS**

Figure 1. Mutation of the TRP box produced functional channels. (A) Amino acid sequence of the TRP domain of TRPV1. In red is highlighted the TRP box sequence, whose amino acids have been individually mutated to A. (B) Images showing the intracellular rise in Ca²⁺ evoked by 1 μM capsaicin from HEK293 cells expressing the TRPV1 and mutant species. (C) Change in Ca²⁺-dependent fluorescence of TRPV1-transfected and mock cells as a function of time, before and after the exposure to 1 (Top) and 100 μM (Bottom) for 10 s, respectively. Representative images and traces of 50 cells measured in n=3 different experiments. Cells were loaded with Fluo4-AM to record intracellular calcium signals and activated as indicated. The extracellular concentration of Ca²⁺ was 2.0 mM.

Figure 2. I696A, W697A and R701A displayed altered capsaicin sensitivity. (A) Dose-response curves for capsaicin activation of mutants. Ionic currents were evoked at +160 mV with a 100 ms voltage pulse. Cells were sequentially exposed to 0.1, 1, 10 and 100 μM capsaicin and depolarized in the presence of the corresponding vanilloid concentration. For TRPV1 the dose response was obtained at -60 mV, since ionic currents evoked with ≥1 μM capsaicin at 160 mV gave rise to sizable currents that could not be clamped properly. Ionic currents were normalized to the estimated maximal current (Iₘₐₓ) obtained from the fit to a Michaelis-Menten binding isotherm to obtain the EC₅₀ (concentration of agonist needed to activate the half-maximal response) and the Hill coefficient of the activation process. (25). The values obtained were: a) TRPV1, EC₅₀= 0.6±0.1 μM, nᵢₜ=1.5±0.1; b) I696A, EC₅₀= 3.6±0.4 μM,
n_H=0.9±0.1; c) W597A, EC_{50}=6.3±0.7 μM, n_H=0.8±0.1; d) R701A, EC_{50}=43±3 μM, n_H=0.7±0.1. (B) Hill coefficient for TRPV1 and mutant channels. (C) Maximal current density obtained for TRPV1 and mutant channels at 160 mV. For TRPV1 current density was measured with 1 μM capsaicin (higher capsaicin concentrations could not be used because of clamping problems), while for mutants a 100μM of the vanilloid was used. Data are given as mean ± SD with, n≥4.

Figure 3. Mutation of the TRP box residues to A produced channels that are not gated by voltage (A) Representative family of whole cell currents elicited with a voltage protocol consisting of 50 ms depolarizing pulses from -120 mV up to 300 mV in steps of 20 mV (inset) for TRPV1, I696A, W697A, K698A, L699A, Q700A, R701A mutants. The holding potential was 0 mV. (B) Current density for wild type and mutants measured at +300 mV. Data are given as mean ± SD, with n≥5.

Figure 4. Mutation of TRP box residues altered the energetics of voltage-dependent channel opening. (A) G-V relationships for TRPV1 wild type and mutant channels. Conductance changes were obtained from the ionic currents using G=I/(V-V_R), where V is the stimulation potential value and V_R is the reversal obtained from the I-V curves. Solid lines depict the best fit to a Boltzmann distribution. (B) V_{0.5} values for the different TRPV1 species obtained from the Boltzmann distribution of G-V relationships. (C) The apparent gating valence of the activation process obtained using the zg=25.69 mV/a_n, a_n is the slope of the G-V curves. (D) Bar graph of the free energy at 0 mV and 25ºC (ΔG_o) for TRPV1 and mutants assuming a two-state model. The ΔG_o was obtained from ΔG_o= zgFV_{0.5}, where F is the Faraday Constant (0.023 Kcal/mol mV), V_{0.5} is the voltage required to activate half-maximal conductance, and zg is the apparent gating valence. All values are mean ± SD with n≥5.

Figure 5. I696A, W697A and R701A mutant channels exhibited voltage-dependent gating in the presence of capsaicin. Ionic currents evoked by increasing concentrations of capsaicin from the I696A (A), W697A (B) and R701A (C) mutants. I-V relationships were recorded using a ramp protocol consisting of a voltage step of 300 ms from 0 mV to -120 mV, followed by a 350 ms linear ramp up to 160 mV. Cells were sequentially exposed to the increasing capsaicin concentrations. Inset in panel (A) depicts the I-V curve for TRPV1 wild type in the absence and presence of 1 μM capsaicin. Representative current traces from n=4 cells measured are shown. Whole cell currents were obtained in symmetrical 150 mM NaCl, from a holding potential of 0 mV. (D-F) G-V relationships for I696A, W697A and R701A mutants, respectively, obtained at increasing capsaicin concentrations. Conductance values were obtained as described in Fig. 4, and fitted to a Boltzmann distribution (solid lines). (G) V_{0.5} values and (H) gating valence (z_g) as a function of the vanilloid concentration for the three mutants. V_{0.5} and z_g were obtained from the G-V curves. (I) Free energy between the closed and open state at 0 mV and 25ºC, obtained as described in Fig. 4. All values are mean ± SD with n≥5.

Figure 6. Mutation of I696 to A notably affected TRPV1 channel properties. (A) Variation of the free energy of channel activation of mutants I696A, W697A and R701A with respect to TRPV1 in the presence of a concentration of capsaicin that matched their respective EC_{50} values. ΔΔG_0=ΔG_0\text{mutant}-ΔG_0\text{TRPV1}. Free energies were obtained as described in Fig. 4. (B) Rectification index of mutants as function of the capsaicin concentration. The rectification index (RI) was calculated as the ratio of the conductance at 150 mV and -100 mV using RI=(I_{150}/(150-V_R))/(I_{-100}/(-100-V_R)). I is the ionic current measured at the indicated potential, and E_R denotes the reversal potential. (C) Kinetics of the activation process at 160 mV for mutants as a function of the vanilloid concentration. The activation process was fitted to one exponential function of the form: I(\hat{I}) = Ae^{-\tau/\hat{I}} where A is the amplitude of the ionic current I, and \tau the time constant of the activation process (SI, Fig. S6). Ionic currents were fitted with the Pulse/PulseFit software. All values are mean±SD with n≥5.
Figure 7. Heat-dependent activation of TRP box mutants. (A) Mean amplitude of the ionic currents evoked by a heat ramp from 25°C to 45°C recorded at +50 mV and 45°C for TRPV1 and chimaeric channels. Ionic currents were obtained from the recordings shown in SI, Fig.S7. (B) I-V relationships for TRPV1 and mutant channels obtained at 45°C. Cells were depolarized from -120 mV to +160 mV in 350 ms. Traces are representative of n≥3 cells. (C) Activating threshold temperature (T) and (D) heat sensitivity (Q₁₀) of channel gating determined at +50 mV for TRPV1 and mutants. The threshold temperature was determined as that which activated 10% of the maximal response. The Q₁₀ factor was obtained for the inverse slope of the log(I) vs. 1/T(ºK) between 35º and 45ºC as described by (35). Data are given as mean±SD, with n≥3 cells. *denotes p<0.05 obtained with the 1-way ANOVA.

Figure 8. Effect of heat on the capsaicin responses of TRPV1 mutants. Mean amplitude of the ionic currents evoked by heat (45°C) and 1 μM capsaicin at 25°C and 45°C for I696A, W697A and R701A. Ionic currents were recorded at +50 mV. Data are given as mean±SD, with n≥3 cells. *denotes p<0.05 obtained with the 1-way ANOVA.

Figure 9. A putative model for the proposed coiled-coil structure of the TRP box in TRPV1. (A) Molecular conservation of the TRP box (Red Box). Multiple sequence alignments of TRPV, TRPM and TRPC families were done with Clustalw (http://www.expasy.org/tools/), showing the high degree of conservation of the TRP box region. (B) Structural arrangement of the TRP box in TRPV1 according to our proposed model for the C-terminus of TRPV1 (19). The TRP box could participate in the formation of a tetrameric coiled-coil structure, with I696 in the bundle core, W697 interacting with adjacent subunits and R701 exposed to the solvent and interacting either with the lipid bilayer or other receptor regions.
Figure 1

A --MGETVNKIAQESKNIWKLQRaitilDTEKSFLKCMR--

B
(ctrl) 1 µM Cap

I696A
W697A
K698A
L699A
Q700A
R701A
TRPV1
MOCK

C

1 µM Capsaicin

MOCK

100 µM Capsaicin

Fluorescence (a.u.)

Time (sec)
Figure 2

Panel A: Graph showing the normalized current (I/I_{max}) against concentrations of capsaicin (μM). Three different lines represent TRPV1, I696A, W697A, and R701A mutants.

Panel B: Bar graph showing the Hill number for TRPV1, I696A, W697A, and R701A mutants.

Panel C: Bar graph showing the maximum current (I_{max}) for TRPV1, I696A, W697A, and R701A mutants.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A

B

C

Figure 8
SUPPLEMENTAL INFORMATION

Experimental procedures

Confocal microscopy. HEK293 cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine and 1% penicillin-streptomycin solution. Before transfection the cells were plated at a density of ~2.5×10⁵ cells/cm² onto poly-D-lysine-coated coverslips for immunofluorescence. Cells were transfected with 2 µg of DNA encoding the TRPV1 and mutant channels and used for experiments 48 hr after transfection. Transfected cells were washed with PBS and fixed with 5% parafomaldehyde/4% sucrose for 20 min at 4°C. Fixing solution was extensively washed out, and the fixed, intact cells were incubated with 5 µg/ml of wheat germ agglutinating conjugated to Alexa Fluor® for 20 min at ≈22°C under continuous agitation and protected from light. The excess of the lectin was washed out with PBS, and cells were permeabilized with 3% horse serum, 2% BSA and 0.1% NP40 in PBS for 20 min at ≈22°C under mild agitation. Thereafter, cells were incubated with anti-TRPV1 (1:1,000; guinea pig, CHEMICON International) overnight at 4°C. After extensive washing, cells were exposed to CyTM-conjugated AffinityPure donkey anti-guinea pig (1:200; Jackson ImmunoResearch) for 1 h at ≈22°C under continuous agitation and protected from light. Cells were embedded and mounted in VECTASHIELD® Mounting medium (VECTOR Laboratories), and analyzed by confocal microscopy in a LSM 5 PASCAL (ZEISS).

Patch clamp measurements in HEK293 cells. HEK293 cells were co-transfected with TRPV1 species and the EYFP protein (pEYFP, Clontech). Membrane currents were recorded with the whole cell configuration using patch clamp as described [1]. For whole cell recordings, pipette solution contained (in mM): 150 NaCl, 3 MgCl₂, 5 EGTA and 10 HEPES, pH 7.2 with CsOH, and bath solution contained (in mM): 150 NaCl, 1 CsCl, 1 MgCl₂, 1.5 CaCl₂, 10 glucose and 10 HEPES, pH 7.4 with NaOH. The different saline solutions were applied with a gravity-driven local microperfusion system with a rate flow of ~200 µl/min positioned at ~100 µm of the recorded cells. All measurements were performed at 20-22 ºC. A voltage step protocol consisting of 50 ms depolarizing pulses from -120 mV to 300 mV in steps of 20 mV was used. The holding potential was 0 mV and the time interval between each pulse was 5s. I-V relationships were studied using a ramp protocol consisting of a voltage step of 300 ms from the holding potential of 0 mV to -120 mV, followed by 350 ms linear ramp up to 160 mV. Time interval between each pulse was 10 s. 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 less than 10 MΩ and to minimize voltage errors was compensated to 70-90%. Reversal potentials were determined from the I-V curves as the potential where I=0. Kinetics of channel activation was calculated by fitting the activation curves to one exponential function of the form: \( I(t) = A e^{-t/\tau} \) where A is the amplitude of the ionic current I, and \( \tau \) the time constant of the activation process. Data were analysed with PulseFit 8.11 (HEKA Elektronik), and Origin 7.0 (MicroCal). Data were expressed as mean±SD, with n=number of cells. Statistical analysis was performed with Student’s t-test and a p <0.05 was taken as the level of significance.

Temperature stimulation. Coverslips with transfected HEK293 cells were placed in a microchamber and continuously perfused (~1ml/min) with solutions warmed at 25°C. The temperature of the solutions was controlled using a CL-100 bipolar temperature controller and a SC-20 dual in-line heater/cooler and was measured by a TA-29 thermistor (Warner Instruments). The temperature probe was placed near the solution outflow and <500 µm of the patch-clamped cell. The time course of the temperature change was ~0.3°C/s. Temperature ramps were first obtained at -60 mV and, thereafter at +50 mV in the same cell. Macroscopic ion currents were recorded with a Multiclamp amplifier using pCLAMP software and a Digidata 1322A digitizer (Axon Instruments; Molecular Devices Corporation). Temperature ramps at -60 and +50mV were sampled at 5 kHz and low pass filtered at 650 Hz. Analysis was performed with pClamp9, WinASCAD software (G. Droogmans, Katholieke Universiteit Leuven, Belgium, ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/winascd.zip) and Origin 7.0 (MicroCal).
Figure S1. Mutation of the TRP box residues does not alter the protein surface expression. (A) Confocal microscopy images of HEK 293 cells transfected with TRPV1 wild type and mutant subunits sequentially exposed to a fluorescently-labelled wheat agglutinin (Alexa-lectin), and to the anti-TRPV1 antibody. (B) Images of cells labelled with the fluorescent lectin and an antibody against calnexin, a marker of the endoplasmic reticulum. Fixed, intact cells were incubated with the fluorescent lectin, and thereafter were permeabilized with detergent and exposed to the anti-TRPV1 antibody.
Figure S2. Analysis of the kinetics of channel activation as a function of the voltage. TRPV1 and mutant channels were stimulated with 100 ms depolarizing voltage steps from +120 mV to +160 mV. The activation process was fitted to one exponential function of the form: \( I(t) = A e^{-t/\tau} \) where A is the amplitude of the ionic current I, and \( \tau \) the time constant of the activation process. Ionic currents were fitted with the Pulse/PulseFit software. Data are given as mean±SD, with n≥5.
Figure S3. Mutation of the TRP box did not affect the reversal potential. The reversal potential was obtained from the corresponding I-V relationships, as that where I=0. For TRPV1, K696A, L699A and Q700A the I-V curves were obtained in the absence of capsaicin. In contrast, for I696A, W697A and R701A the reversal potential was obtained from I-V elicited in the presence of 10 μM capsaicin. Data are given as mean±SD, with n≥5.
Figure S4. Analysis of the activation kinetics of mutants as a function of the capsaicin concentration. Mutant channels were stimulated with 100 ms depolarizing voltage steps from -120 mV to +160 mV in the absence and presence of capsaicin at different concentrations (1-100 μM). Cells were sequentially exposed to the increasing concentrations of the vanilloid. (Inset) Activation curves of TRPV1 in the absence and presence of 0.1 μM capsaicin. The activation process was fitted to one exponential function of the form: \( I(t) = A e^{-t/\tau} \) where \( A \) is the amplitude of the ionic current \( I \), and \( \tau \) the time constant of the activation process. Ionic currents were fitted with the Pulse/PulseFit software. Time constant values of the traces at +160 mV are displayed.
Figure S5. Heat-dependent gating of TRPV1 and mutant channels. Traces represent the ionic currents evoked by heat ramps (depicted on top, 0.3°C/s) from cells held at -60 mV (left panels) and +50 mV (right panels). Traces are representative of n≥3 cells.
REFERENCES
