Biochimica et Biophysica Acta 1833 (2013) 520–528

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Protons stabilize the closed conformation of gain-of-function mutants of the TRPV1 channel

Stepana Boukalova, Jan Teisinger, Viktorie Vlachova *

Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic

ARTICLE INFO

Article history:
Received 27 August 2012
Received in revised form 29 October 2012
Accepted 20 November 2012
Available online 4 December 2012

Keywords:
Vanilloid receptor subtype 1
Transient receptor potential
Gating
Tryptophan-scanning
Mutagenesis

ABSTRACT

The vanilloid transient receptor potential channel TRPV1 is a molecular integrator of noxious stimuli, including capsaicin, heat and protons. Despite clear similarities between the overall architecture of TRPV1 and voltage-dependent potassium (Kv) channels, the extent of conservation in the molecular logic for gating is unknown. In Kv channels, a small contact surface between S1 and the pore-helix is required for channel functioning. To explore the function of S1 in TRPV1, we used tryptophan-scanning mutagenesis and characterized the responses to capsaicin and protons. Wild-type-like currents were generated in 9 out of 17 mutants; three mutants (M445W, A452W, R455W) were non-functional. The conservative mutation R455K in the extracellular extent of S1 slowed down capsaicin-induced activation and prevented normal channel closure. This mutant was neither activated nor potentiated by protons, on the contrary, protons promoted a rapid deactivation of its currents. Similar phenotypes were found in two other gain-of-function mutants and also in the pore-helix mutant T633A, known to uncouple proton activation. We propose that the S1 domain contains a functionally important region that may be specifically involved in TRPV1 channel gating, and thus be important for the energetic coupling between S1–S4 sensor activation and gate opening. Analogous to Kv channels, the S1-pore interface might serve to stabilize conformations associated with TRPV1 channel gating.

1. Introduction

Transient Receptor Potential vanilloid receptor-1 (TRPV1) is a nonselective cation channel that is gated in response to various noxious stimuli: pungent vanilloids (capsaicin), noxious heat (>42 °C), and low pH (<6.8). The polymodal gating of this channel is a crucial mechanism contributing to the function of nociceptive neurons. Although numerous studies have been performed with recombinant TRPV1 since its cloning [1], its gating properties are not well understood. The molecular architecture of the transmembrane region of TRPV1 is thought to be potentially analogous to the large family of voltage-dependent Kv potassium channels [2–5]. Each subunit of the homotetrameric TRPV1 channel contains six transmembrane domains (S1–S6) with the S1–S4 sensor region harboring the capsaicin binding site centered on Y511/S512 between the S2 and S3 helices [6], and with a pore domain formed by S5, S6, and the loop between them (S5–P-S6) [7]. Similar to Kv channels, the conformational changes within the S1–S4 sensors are most likely converted by the inner S4–S5 linker helices directly into gate opening and closing through the motions of the S6 inner helices which dilate (open) and constrict (close) the pore entryway [8–12]. Indeed, our recent study showed that single point mutations R557K and G563S in the S4–S5 linker of TRPV1 resulted in gain-of-function phenotypes so that they stabilized the open conformation and led to overactive channels [13]. However, analogous to potassium (Kv) channels, a second protein interface between the S1–S4 sensor and the pore might exist that serves to fix the sensor's position relative to the pore and affect the gating [14]. In Kv channels, the extracellular extent of S1 encompasses co-evolved residues that make physical contact with the pore-helix over a small area near the extracellular membrane surface. The existence of such a second putative protein-protein contact in TRPV1 is implicitly supported by recent findings that proton activation and potentiation involves not only the titratable pore residues E600 and E648, but also the peripheral domains S1–S4 [15,16].

In this study, we sought to explore the possibility that the S1 domain contains a functionally important region that may be specifically involved in TRPV1 channel gating, and thus be important for the energetic coupling between S1–S4 sensor activation and gate opening. We identified several residues that, when mutated, produced gain-of-function phenotypes with an increased basal channel activity and impaired ability to close after capsaicin was washed out.

* Corresponding author at: Department of Cellular Neurophysiology, Institute of Physiology AS CR, Vídenská 1083, 142 20 Prague 4, Czech Republic. Tel.: +420 29644 2711; fax: +420 29644 2488.
E-mail address: vlachova@biomed.cas.cz (V. Vlachova).
URL: http://www2.biomed.cas.cz/d331/ (V. Vlachova).

0167-4889/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2012.11.017

Surprisingly, we found that protons neither activate nor potentiate these mutants and, instead, promote rapid deactivation of the currents.

2. Materials and methods

2.1. Preparation of TRPV1 constructs and cell culture

HEK293T cells were cultured in OPTI-MEM I medium (Invitrogen) supplemented with 5% FBS. Cells were transiently transfected with a cDNA plasmid encoding wild-type or mutant rat TRPV1 and eGFP (TaKaRa) using the Magnet-assisted Transfection (IBA GmbH) method. TRPV1 cDNA in the pcDNA3 vector was kindly provided by David Julius (University of California, San Francisco, CA). Cells were used 24–48 h after transfection. At least three independent transfections were used for each experimental group. The TRPV1 constructs were generated using a QuikChange XL Site-directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing (ABI PRISM 3100; Applied Biosystems).

2.2. Electrophysiology and experimental solutions

Patch-clamp experiments were performed using an Axopatch 200B amplifier controlled by pCLAMP 10 software (Molecular Devices) in whole-cell voltage clamp configuration. Data were filtered at 2 kHz (−3 dB, four-pole Bessel filter) and digitized at 4–20 kHz. Only one recording was performed on any one cover slip of cells to ensure that recordings were made from cells not previously exposed to the agonist. The extracellular control solution contained 160 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, adjusted to pH 7.3 with NaOH, osmolarity 325 mOsm. In order to prevent desensitization, the chemical-induced activity of TRPV1 was studied in Ca^{2+}-free solution, in which $CaCl_2$ was replaced with 1 mM EGTA. In a solution of pH 5.5, MES buffer was used instead of HEPES. The pipette solution contained 125 mM cesium gluconate, 15 mM CsCl, 5 mM EGTA, 10 mM HEPES, 0.5 mM $CaCl_2$, 2 mM MgATP, pH 7.3, osmolarity 283 mOsm. All chemicals were purchased from Sigma-Aldrich. Solutions were applied to cells using a fast superfusion system [17].

2.3. Data analysis

All data were analyzed using pCLAMP 10.2 (Molecular Devices), and curve fitting and statistical analyses were done in pCLAMP 10.2 and SigmaPlot 10 (Systat Software). No leak subtraction was utilized. To evaluate the kinetics of capsaicin-induced responses, the onset phase of capsaicin-evoked currents was fitted to the double exponential function $I(t) = A_{fast} \cdot \exp(-t/\tau_{fast}) + A_{slow} \cdot \exp(-t/\tau_{slow}) + C$, where τ_{fast} and τ_{slow} are the time constants of the fast and slow components of activation, A_{fast} and A_{slow} are the amplitudes of each component and C is a constant. The kinetics of capsaicin washout were determined as the time taken for the current to decrease to 50% of its level before removing the agonist. Voltage-dependent gating parameters were estimated from steady state conductance–voltage (G–V) relationships obtained at the end of 100-ms voltage steps by fitting the conductance $G = I/(V - V_{rev})$ as a function of the test potential V to the Boltzman equation: $G = ((G_{max} - G_{min})/(1 + \exp[-zF(V - V_{1/2})/RT])) + G_{min}$, where z is the apparent number of gating charges, $V_{1/2}$ is the half-activation voltage, G_{min} and G_{max} are the minimum and maximum whole-cell conductance, V_{rev} is the reversal potential, and F, R, and T have their usual thermodynamic meaning. All data are expressed as mean ± SEM. from n independent experiments. Overall statistical significance was determined by the analysis of variance. When significance was found ($P<0.05$), statistical comparisons were performed using Student's t test or the Mann–Whitney U test for individual groups.

3. Results

3.1. Gain-of-function mutations in S4–S5 region of TRPV1 are inhibited by protons

In a recent study by ourselves, we described two overactive mutant TRPV1 ion channels with a substitution in the fourth transmembrane segment and the S4–S5 linker (R557K and G563S). When measured at a holding potential of −70 mV using the whole-cell voltage clamp technique, the basal activity of these mutants was increased and the kinetics of their capsaicin-evoked responses was markedly slowed. In many HEK293T cells which heterologously expressed R557K mutant channels, and in all G563S-expressing cells, deactivation after capsaicin washout was incomplete [13] (Fig. 1). We were interested in whether these gain-of-function mutants respond to low pH similarly to wild-type TRPV1.

To determine pH sensitivity, we measured the whole-cell inward currents evoked by consecutive applications of pH 5.5 and a saturating concentration of capsaicin (1 μM; applied until the maximum current amplitude was reached) in HEK293T cells expressing wild-type, R557K or G563S mutant TRPV1 channels (Fig. 1A to C). To desensitize the endogenous acid-sensing ion channels, we pre-applied the extracellular solution adjusted to pH 6.8 for 20 s prior to the application of a solution of pH 5.5, as described in [18]. In wild-type TRPV1, pH 5.5 evoked currents of approximately half the magnitude of the maximal response induced by capsaicin (52.1 ± 6.4%; $n = 12$). In contrast, R557K and G563A could not be activated by lowering the pH of the extracellular solution; on the contrary, the application of pH 5.5 resulted in a decrease in the initial basal current of mutant channels to −0.7 ± 0.5% ($n = 9$) and −2.4 ± 1.9% ($n = 6$) of the maximal response induced by capsaicin (Fig. 1C). We observed that even the pre-application of a mildly acidic pH (6.8) by itself was able to reduce the basal currents to 76.6 ± 10.0% of the initial level in R557K and to 67.4 ± 4.3% in G563S. Most notably, when a mildly acidic solution (pH 6.8) was applied during the washout phase after capsaicin treatment, the inward currents through these mutant channels were rapidly deactivated (Fig. 1A). This inhibitory effect of protons was not readily reversible, so that no currents were induced after washing out the acidic solution, and subsequent application of 1 μM capsaicin exhibited a slowed activation kinetics, indicating that protons do not inhibit TRPV1 mutants by a simple pore blocking mechanism [19].

Protons activate and sensitize wild-type TRPV1 by binding to two extracellularly located acidic residues (E600 and E648) in the pore region (Fig. 1D) [20]. More recently, it has been found that peripheral domains of TRPV1 also play an important role in transducing the pH signal to channel opening [15,16]. Analogous to Kv channels, the extracellular extent of TRPV1-S1 could make physical contact with the pore region and transmit the conformational change evoked by protonation to peripheral domains [14]. The role of S1 in TRPV1 had not yet been studied, therefore we decided to explore its function using tryptophan-scanning mutagenesis.

3.2. Tryptophan perturbation scan reveals strongly affected TRPV1 phenotypes and indicates an α-helical structure of S1

The residues in the putative first transmembrane segment (S1) of the rat subtype of TRPV1 (residues F439 to R455) were replaced, one by one, with tryptophan, in order to determine which regions are important for ion channel functioning. The aromatic residues were replaced by non-aromatic residues to impair their potential interactions with the membrane interface or other amino acid side chains in the protein complex. The results of the mutagenesis analysis of S1 are summarized in Fig. 2. Our data quantifying the maximal capsaicin-induced currents at −70 mV revealed a clear α-helical periodicity of the upper part of S1, with mutation-sensitive and insensitive residues at opposite sites of the helix projection (Fig. 2A). For each mutant, we tested

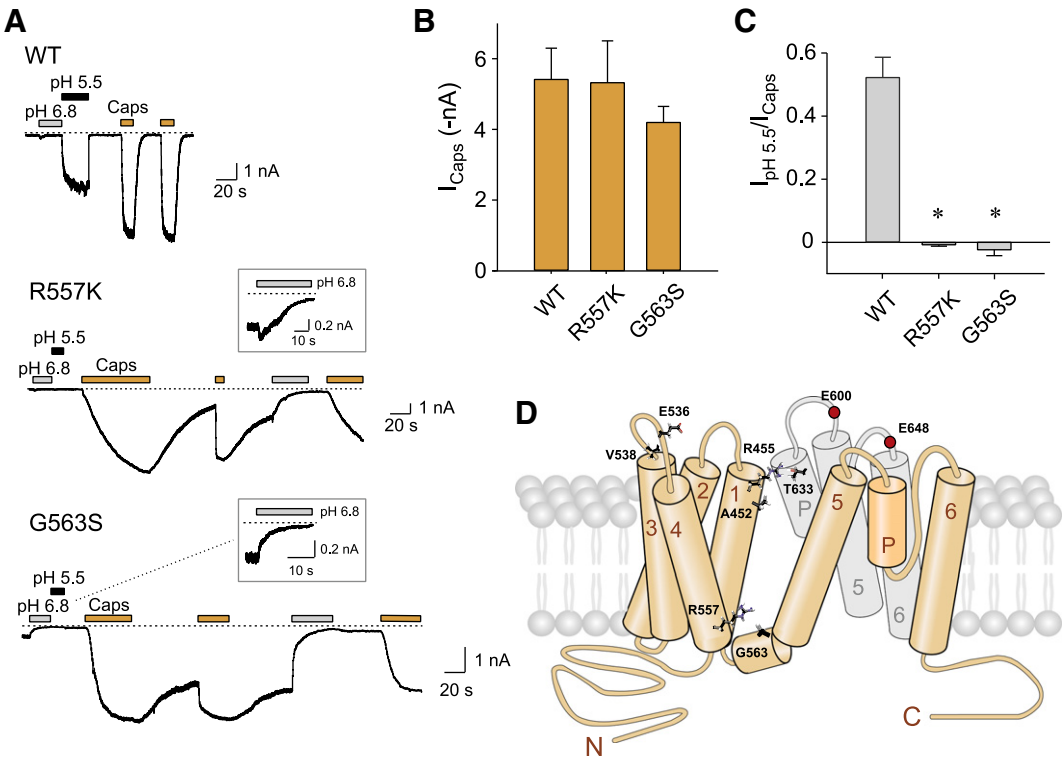

Fig. 1. TRPV1 mutants R557K and G563S are not activated by reducing extracellular pH. (A) Representative recordings of whole-cell current responses of HEK293T cells expressing rTRPV1 evoked by pH 5.5 and 1 μM capsaicin. To desensitize endogenous acid-sensing ion channels, we applied a solution of pH 6.8 prior to applying the pH 5.5 experimental solution. Holding potential −70 mV. Note the inhibitory effect of protons (pH 6.8) on TRPV1–R557K and G563S channel function, shown in insets. (B) Summary of capsaicin-induced inward current amplitudes for wild-type and mutant TRPV1. No significant changes were detected (n = 10–55; P = 0.660; statistical analysis was performed using one-way ANOVA on ranks). (C) Effect of R557K and G563S mutations on pH 5.5-evoked current normalized to responses to 1 μM capsaicin. The amplitude of the initial basal current was taken as a baseline. Protons do not activate mutant ion channels. Conversely, low pH reduces the spontaneous activity of TRPV1–R557K and G563S (n = 6–13; *P<0.001; statistical analysis was performed using the Mann–Whitney U test). (D) Cartoon model of TRPV1 subunit depicting those amino acid residues which are important for ion channel functionality. Red dots indicate putative positions of acid-sensing residues (E600 and E648). All data are represented as mean ± SEM.

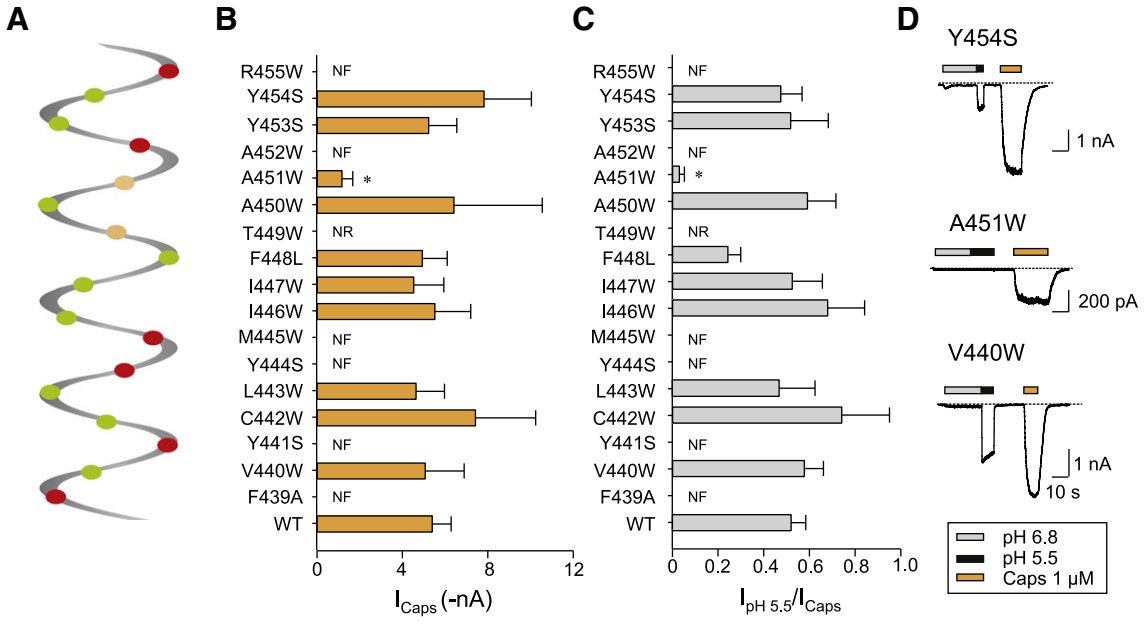

Fig. 2. Tryptophan-scanning mutagenesis of S1 transmembrane region. (A) Putative α-helical topology of TRPV1–S1 segment. Green symbols indicate positions which are insensitive to mutagenesis. Orange symbols indicate residues which, when mutated, strongly alter TRPV1 channel functionality. Red symbols indicate nonfunctional (NF) mutants. (B) Bar graph summarizing mean whole-cell inward current amplitudes of responses evoked by 1 μM capsaicin (n = 4–31) and (C) normalized responses to stimulation by pH 5.5 (n = 4–12) in wild-type and mutant TRPV1. NR marks mutant ion channel in which we only observed measurable responses at positive membrane potentials. Statistical analysis was performed using the Mann–Whitney U test and t test (*P ≤ 0.001). All values are shown as mean ± SEM. (D) Representative whole-cell current responses of Y454S, A451W and V440W mutants evoked by application of low pH and 1 μM capsaicin. Holding potential −70 mV.

its maximal responsiveness to 1 μM capsaicin (Fig. 2B) and sensitivity to low pH (pH 5.5; Fig. 2C). Three aromatic residues in the intracellular portion of S1 (F439, Y441 and Y444) were found to be crucial for TRPV1 channel functioning. Their replacement with small non-aromatic amino-acid side chains (alanine or serine) did not generate functional channels. The tryptophan scanning analysis revealed several other positions in TRPV1-S1 which were sensitive to mutagenesis. Three tryptophan mutants (M445W, A452W and R455W) failed to generate functional channels and the T449W mutant produced only small currents at positive membrane potentials.

3.3. Requirement for small side-chain residues at A451 and A452, and for a positively charged residue at R455

According to a study by Lee et al. [14], the pore region of Kv channels interacts with the first transmembrane segment near the extracellular surface, therefore we chose the A452 and R455 amino acid residues in the extracellular portion of TRPV1-S1 for additional analysis. In addition, we tested the properties of the side chain at position 451, where replacing the native alanine residue with tryptophan significantly reduced the amplitude of the responses evoked both by capsaicin and low pH. Fig. 3 summarizes the results obtained from the mutagenesis analysis of the properties of the side chains at positions A451, A452 and R455. The introduction of small residues (serine or valine) at position 451 compensated the diminished responses to both capsaicin and pH 5.5. In contrast, replacing the subsequent alanine at position 452 with valine was not able to recover the impaired functioning of TRPV1. The charge-neutralizing mutation at R455, which is located approximately one helix turn upstream from A452, led to a complete loss of function. In contrast, the charge-conserving mutation R455K did not affect the magnitude of capsaicin-evoked responses ($P=0.167$; $n=22$; Mann–Whitney U test) but completely abolished the pH-induced activity. pH 5.5 inhibited R455K-mediated currents to $-0.9\pm0.6\%$ of the maximal response induced by 1 μM capsaicin, measured from the basal level ($n=4$; Fig. 3B), indicating that a charged residue at position 455 is important for TRPV1 channel functioning, and even the small dislocation caused by the replacement of arginine with the similar amino acid lysine was able to impair pH-dependent channel gating.

According to the homology model [11], two glutamate side chains are located near the arginine R455 in the upper part of S1 and are possible interaction candidates: the first, E458, located three amino acid residues upstream from R455, and the second, E478, in the putative upper part of the second transmembrane segment of the same TRPV1 subunit. To probe these putative interactions, we introduced charge-neutralizing (E478A) and charge-reversing (E458K) mutations at these two positions and assessed the functional properties of the constructs (Fig. 3). The capsaicin and low-pH-evoked responses from E458K and E478A-expressing cells were not significantly different from wild-type TRPV1, which argues against a direct intramolecular interaction of R455 with E458 or E478. This may indicate that the small alanine residue at position 452 and the positively charged residue R455, both on one face of the S1 α-helical wheel, comprise an interaction surface that is complementary to other parts of the TRPV1 channel protein complex.

3.4. R455K and T633A display overactive phenotypes similar to those of the gain-of-function mutations in the S4–S5 region

The R455K mutation completely abolished the proton-induced currents while preserving the maximum capsaicin-evoked responses, similar to what has been previously reported for the V538L and T633A mutants of rat TRPV1 [15]. These latter two residues have been proposed to be involved in the transduction of the protonation signal into channel opening. Therefore, we were further interested in whether mutations which selectively abrogate low-pH-induced currents generally affect the kinetics of capsaicin responses, as we also observed in the pH-insensitive R557K and G563S overactive

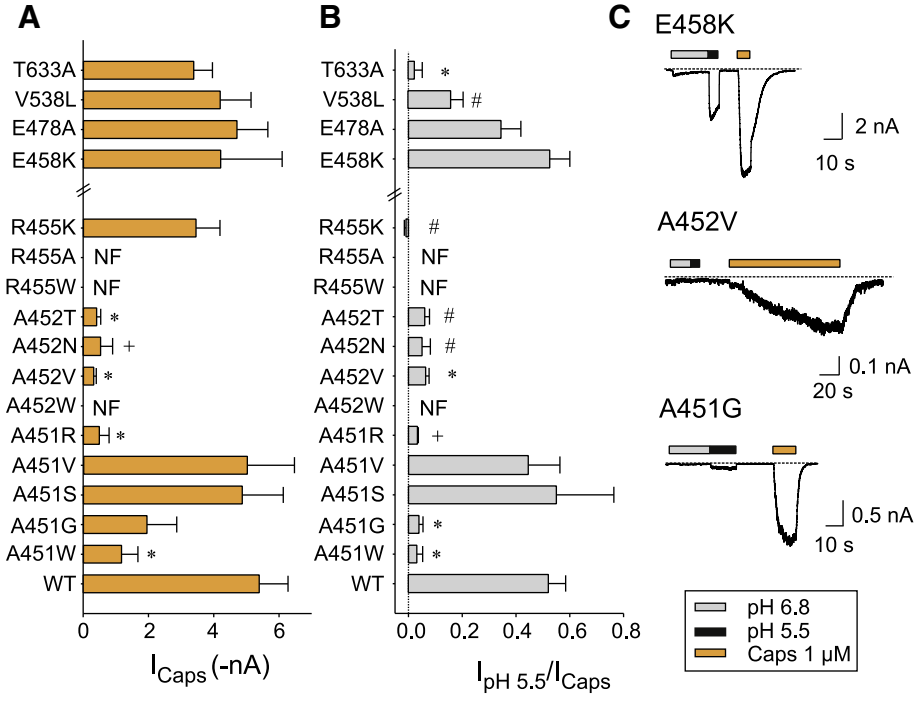

Fig. 3. A positively charged residue at position 455 is required for TRPV1 channel functionality. (A) Mean amplitudes of whole-cell inward current evoked by application of 1 μM capsaicin ($n=3$–31) and (B) responses to stimulation by pH 5.5 normalized to capsaicin-induced responses ($n=3$–12) for several TRPV1 ion channels with mutations in upper part of S1 (A451, A452, R455) and for some constructs with mutated residues which could potentially interact with S1 (E458, E478, V538, T633). The R455K mutation uncouples proton activation from capsaicin-induced activation. As reported in ref. [15], a similar phenotype is observed in V538L and T633A constructs. Statistical analysis: Mann–Whitney U test and t test; *$P<0.001$; #$P<0.01$; +$P<0.05$. All data are represented as mean ± SEM. (C) Representative whole-cell current responses of E458K, A452W and A451G mutants evoked by application of low pH and 1 μM capsaicin. Holding potential −70 mV.

mutants (Fig. 1A). We compared the kinetic parameters of the current responses to two consecutive applications of 1 μM capsaicin in wild-type TRPV1 and in the five mutants in which low pH sensitivity was found to be uncoupled from capsaicin responsiveness (Fig. 4). A two-exponential fit of the time course of current activation was performed to accurately describe the combination of the fast and slow component of the onset of the first and second capsaicin response.

We found that, except for V538L, all other mutants led to a marked slowing of the activation and deactivation rates of the first capsaicin

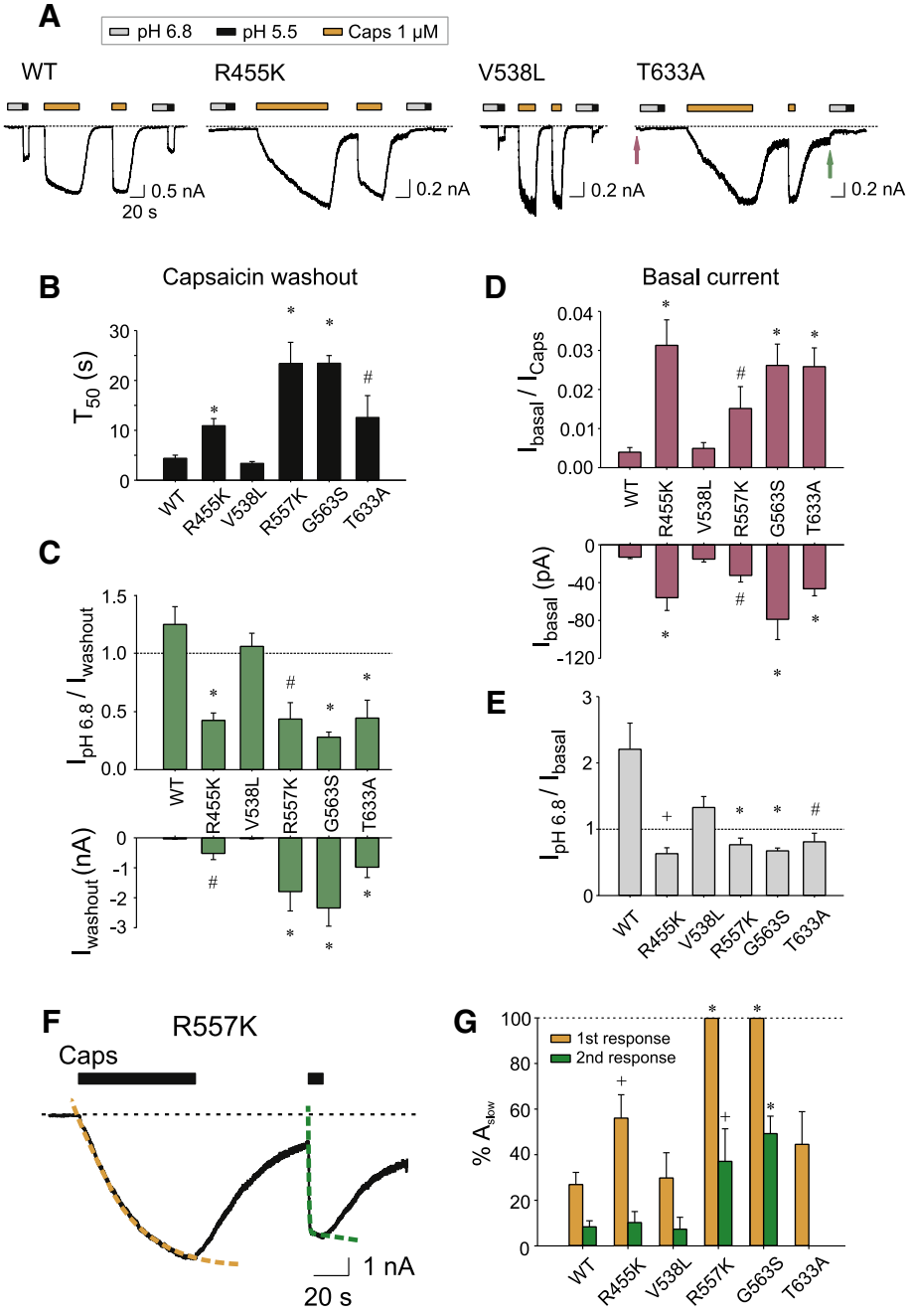

Fig. 4. Protons stabilize closed state in R455K and T633A-TRPV1, similar to R557K or G563S mutant ion channels. (A) Representative whole-cell current responses of wild-type TRPV1, R455K, V538L and T633A mutants evoked by application of low pH and 1 μM capsaicin. Holding potential −70 mV. (B) Effect of mutations on kinetics of capsaicin-induced response offset in wild-type TRPV1, R455K, V538L and T633A mutants. T_{50} represents the time taken for the current to decrease to 50% of its level before removing the capsaicin. For comparison, we included the data from the R557K and G563S mutants, in which the offset of capsaicin-evoked response is significantly slowed ($n=9$–30). (C) Bar graphs summarizing the effect of protons on the inward current remaining after capsaicin washout (upper plot) and the mean amplitudes of this current for the indicated mutants (lower plot; green arrow in panel (A)). Statistical analysis was performed using ANOVA on ranks followed by the *t* test or Mann–Whitney *U* test; $n=5$–24. (D) Summary of relative and absolute value of basal current ($n=13$–81). The amplitude of the basal current was determined in control extracellular solution prior to the application of any agonist (pink arrow in panel (A)). (E) Quantification of inhibitory effect of protons (pH 6.8) on basal current in wild-type TRPV1 and indicated mutants ($n=3$–21). Statistical analysis was performed using the Mann–Whitney *U* test and *t* test; $^{*}P<0.001$; $\#P<0.01$; $+P<0.05$. All data represent mean ± SEM. (F) Representative whole-cell current responses of the TRPV1–R557K mutant evoked by two consecutive applications of 1 μM capsaicin. The onsets of capsaicin responses were fitted with the second-order exponential function, which can reproduce the combination of the fast and slow component of the capsaicin onset. (G) Bar graph summarizing percent representation of the slow component of the first and second capsaicin responses ($100 \times A_{slow}/(A_{slow}+A_{fast})$) for wild-type TRPV1 and indicated mutants. Statistical analysis was performed using Mann–Whitney U test; $^{*}P<0.001$; $+P<0.05$. All data represent mean ± SEM.

response. In R455K, the slow component of the onset of the first capsaicin response ($100 \cdot A_{slow}/(A_{slow}+A_{fast})$) represented 56.0 ± 10.3% ($n=16$) of the whole inward current, which was significantly more than in wild-type TRPV1 (26.9 ± 5.3%; $n=36$; $P=0.014$). Although the T633A construct exhibited a similar ratio between the slow and fast components of the onset of the first capsaicin response to the wild-type (44.5 ± 14.3%; $n=9$; $P=0.270$), its slow component (τ_{slow}) was markedly decelerated (73.7 ± 8.8 s; $n=5$) relative to the wild-type ($\tau_{slow}=22.4\pm4.2$ s; $n=18$; $P=0.002$). Upon capsaicin washout, the half-decay time was slowed in both the R455K ($T_{50}=10.9\pm1.4$ s; $P<0.001$) and the T633A mutant ($T_{50}=12.6\pm4.4$ s; $P=0.003$), compared to the wild-type ($T_{50}=4.4\pm0.6$ s).

Similar to the gain-of-function mutants with a substitution in the S4–S5 region, the R455K and T633A constructs often displayed incomplete deactivation after 40–80 s capsaicin washout and accelerated response to the second application (Fig. 4). The remaining current after capsaicin washout was partially blocked by lowering the pH of the extracellular solution from pH 7.3 to 6.8 (Fig. 4A and C). An acidic solution diminished the inward steady-state current after capsaicin washout to 42.3 ± 6.4% in R455K ($n=18$), to 43.5 ± 14.1% in R557K ($n=9$), to 27.9 ± 4.4% in G563S ($n=24$) and to 44.3 ± 15.4% in T633A ($n=13$). The currents did not increase after restoring the control solution to pH 7.3, indicating that the inhibitory effect of protons is not readily reversible (compare Figs. 1A and 4A). On the contrary, wild-type TRPV1 and V538L channels fully deactivated and mildly acidic pH (pH 6.8) slightly potentiated their currents after 20–30 s of capsaicin washout (by 24.8 ± 15.2% in wild-type and by 5.8 ± 11.3% in V538L; $n=19$ and 5).

In the absence of capsaicin and at 25 °C, wild-type TRPV1 displays only negligible currents at −70 mV and the mutants which exhibit deficiencies in some aspects of channel activation often produce constitutively active channels [21]. To analyze the extent of basal activity in the low-pH-insensitive mutants, we measured the inward current amplitudes at −70 mV before the application of any agonist and compared them with the currents induced by 1 μM capsaicin (Fig. 4D). The amplitude of the basal current was enhanced in cells expressing R455K, R557K and T633A, similar to what we previously also observed with G563S [13]. The application of an acidic solution of pH 6.8 potentiated the basal currents in wild-type TRPV1 to 221 ± 39% (n = 10). However, it significantly inhibited R455K (to 63.1 ± 8.9%; $n=3$) and T633A (to 81.0 ± 13.0%; $n=8$), suggesting that protons change the basal open–closed equilibrium by promoting the closed state of these mutant channels.

3.5. Overactive mutants exhibit altered functional properties in terms of proton-mediated potentiation, voltage sensitivity and heat activation

It is well known that protons at concentrations that do not evoke a marked activation of TRPV1 strongly potentiate the responses induced by low concentrations of capsaicin [22]. In some cases, point mutations that eliminate proton-induced activation do not alter the potentiating effect of low pH [15,20]. To test the extent of proton-mediated potentiation in the mutants described here, we applied a sub-saturating concentration of capsaicin (0.3 μM) adjusted to pH 7.3 and pH 6 (Fig. 5A and B). In accordance with the data published by Ryu et al. [15], proton-mediated potentiation was fully preserved in the V538L mutant (458.9 ± 93.8%; $n=8$) compared to wild-type TRPV1 (358.1 ± 43.5%; $n=11$; $P=0.174$). In a striking contrast, protons inhibited the capsaicin-induced steady-state currents in R455K, R557K and G563S to 58.9 ± 5.3% ($n=6$), 81.7 ± 9.3% ($n=4$) and 60.1 ± 6.6% ($n=4$), respectively. The T633A mutant was also less pH-sensitive than the wild-type, exhibiting only a 140.7 ± 19.5% increase in its capsaicin response at pH 6 ($n=6$; $P=0.003$). These data reveal residues in the S1 and S4–S5 region of TRPV1 that, when mutated, abolish pH-induced activation and potentiation, and even cause an inhibition of capsaicin-induced activation by protons.

Interestingly, a similar but less pronounced inhibitory effect of protons has been recently reported for the F660S mutation in the S6 region of human TRPV1 [23], and this finding led the authors to conclude that protons activate and potentiate TRPV1 by a leftward shifting of the voltage dependence of the activation curve. An overactive mutant ion channel is expected to have an already shifted voltage dependence because of the altered equilibrium between the opened and closed state. Indeed, we found that the conductance-to-voltage relationships were dramatically shifted towards less depolarizing potentials in R455K by 42.4 ± 3.7 mV ($n=25$), in R557K by 79.0 ± 2.3 mV ($n=40$), in G563S by 101.5 ± 2.2 mV ($n=25$) and in T633A by 45.1 ± 2.9 mV ($n=28$) (Figs. 5C and D). The steep temperature-dependence was abolished in all constructs with apparently increased voltage sensitivity (Figs. 5E and F), which could be a result of the already heightened activity of the mutant ion channels at room temperature.

3.6. pH-dependent inhibitory pathway is distinct from proton-induced activation mechanism

Our data show that low pH does not increase the activity of the R455K, R557K and G563S gain-of-function mutant ion channels; conversely it stabilizes their closed conformation. The mechanism by which protons promote channel closure is unclear. One of the probable mechanisms is that the conformational change caused by protonation of the extracellular acidic residues in the pore domain (E600 and E648) [20] affects the gating of the TRPV1 mutant ion channels through S1, and perhaps through interactions involving R455. To test this hypothesis, we generated TRPV1 constructs with the double mutations R455K/E600A and R455K/E648A, which simulate the protonated state of TRPV1, and tested their responses to capsaicin (1 μM) and low pH (6.8) (Fig. 6). Likewise in R455K-expressing cells, we observed an incomplete washout of capsaicin-evoked responses and the remaining inward current was nearly completely blocked by the application of pH 6.8 in both R455K/E600A (to 11.6 ± 5.0%; $n=5$) and R455K/E648A (to 18.3 ± 9.6%; $n=3$). These data indicate that the inhibitory effect of protons on the activity of the gain-of-function mutants is mediated by other pathways than the low-pH-induced stimulatory effects observed in wild-type TRPV1.

4. Discussion

Our results provide initial experimental insights into the possible structural interactions involved in the proton-dependent activation pathway of TRPV1. This ion channel is prototypically polymodal, endowed with the capability to integrate diverse types of cues that are likely to produce conformational changes throughout the whole channel protein complex and substantial crosstalk between the extracellular, transmembrane and cytoplasmic domains [24,25]. Previous studies have identified several sensory regions that are more susceptible than others to capsaicin [6], temperature [26–29], voltage [13] or protons [15,16,20,23,30]. However, even though the proton-dependent activation pathway is among the best studied, no domain interactions that underlie the transition from the resting to activated state have been put forward. What is accepted from research carried out to date is that this process is triggered via two glutamic residues: E600, located on the extracellular side of S5, and E648, positioned in the linker between the selectivity filter and S6 [20]. The downstream activation pathway involves T633 in the pore helix, E536 and V538 in the extracellular loop between S3 and S4 [15,16], and converges at F659 in S6 (F660 in the human orthologue) [23]. In addition, protons most likely induce dynamic changes in the pore region leading to the reduction of unitary conductance by protonating E636 located in the pore helix and D646 at the extracellular pore entrance [15,19,30,31], however, at the whole-cell level, this effect is outweighed by an increase in the probability of opening [19].

By examining specific mutations in the S4–S5 linker of TRPV1, we found that protons neither activate nor potentiate the mutant channels,

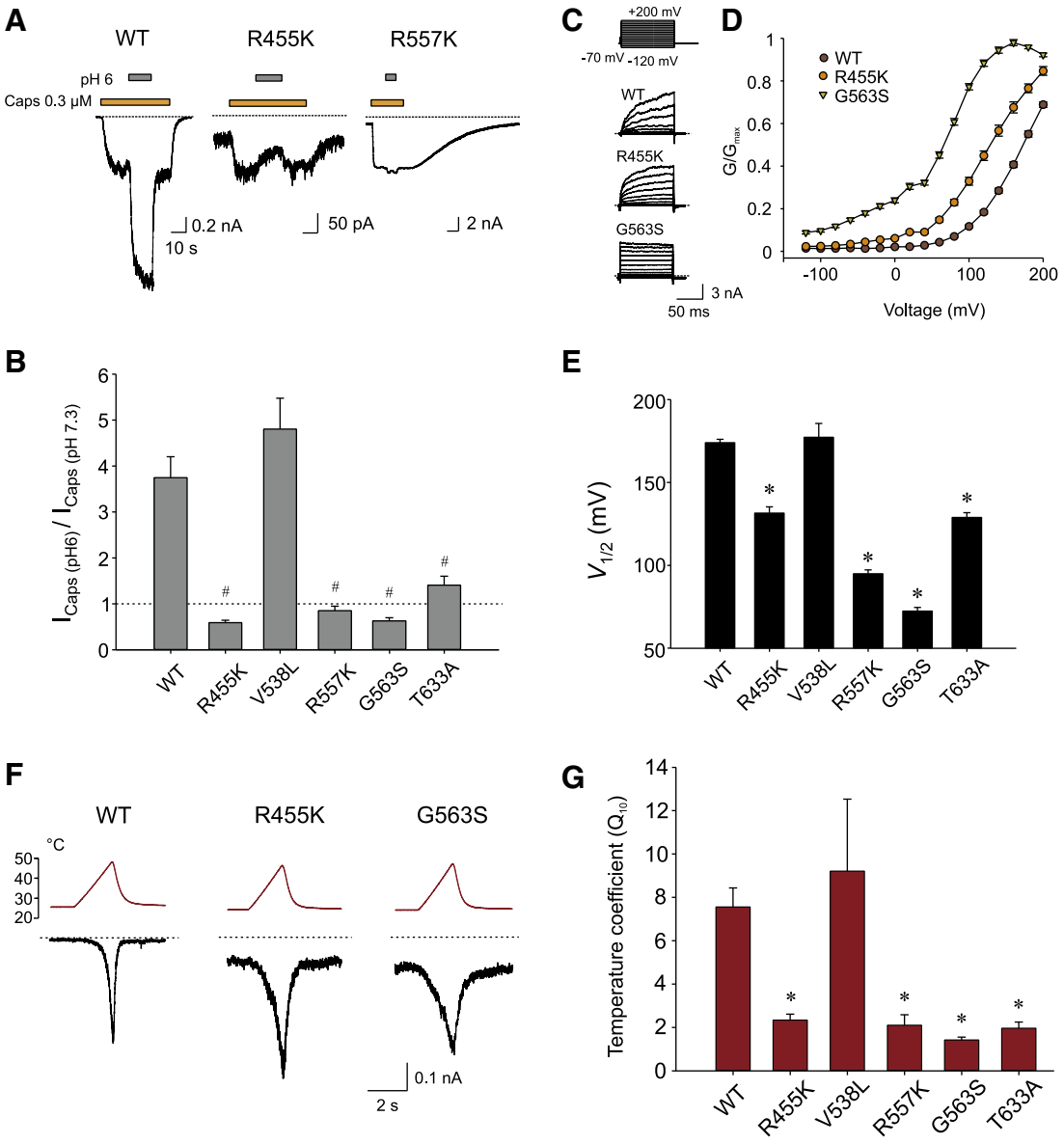

Fig. 5. R455K, R557K, G563S and T633A mutants display impaired proton-induced potentiation and have higher apparent voltage sensitivity. (A) Sample recordings of whole cell current responses to application of experimental solution containing 0.3 μM capsaicin adjusted to pH 7.3 and pH 6 respectively. The currents induced by lowering the pH were recorded in the presence of 50 μM amiloride. Holding potential −70 mV. (B) Bar graph summarizing effect of low pH (pH 6) on capsaicin-induced responses. Statistical analysis was performed using the Mann–Whitney *U* test; #$P<0.01$; $n=4$–11. (C) Representative current traces in response to family of 100 ms voltage steps from −120 mV to 200 mV. (D) Normalized conductance–voltage relationship for wild-type TRPV1, R455K and G563S mutants ($n=24$–58). The data were obtained using the same voltage step protocol as shown in (C). (E) $V_{1/2}$ for wild-type TRPV1 and indicated mutants (*t* test; *$P<0.001$; $n=7$–58). (F) Representative recordings of whole-cell current responses to application of 22–48 °C heat ramp (10 °C/s) at −70 mV for wild-type, R455K and G563S mutant ion channels. (G) Summary of temperature coefficients (Q_{10}) for indicated constructs. Q_{10} was determined from ratio of current amplitudes at 45 °C and 42 °C. Statistical analysis was performed using Kruskal–Wallis one way analysis of variance on ranks and Mann–Whitney *U* test; *$P<0.001$. All data represent mean ± SEM.

but instead stabilize their resting conformation. Given the crucial role of the outer pore region in proton-mediated modulation, together with the important role of the S4–S5 region in voltage-dependent gating [13] and the fact that proton sensing in TRPV1 is associated with a shift in its voltage dependence [23], we inferred that there might be at least a functional link between the S1–S4 sensor module and the outer pore that serves to regulate the gating. We performed a systemic tryptophan-perturbation scan in the S1 domain of rat TRPV1 to probe the orientation and structural basis of the ability of S1 to affect the activation kinetics of the channel and, thus, to explore the extent of conservation between the molecular logic for gating between TRPV1 and potassium Kv channels [14]. The underlying assumption of the tryptophan (Trp) perturbation mutagenesis approach is that replacing the residue with tryptophan, which contains a bulky hydrophobic side chain, should not be tolerated in positions involved in tight protein-protein interactions and tolerated in positions that interact with lipids or participate in loose protein-protein interactions [32]. Using this approach, we identified six out of 17 mutants that failed to generate currents in response to capsaicin, depolarizing voltage and low pH, indicating that the environment surrounding S1 is likely to have a significant impact on TRPV1 functioning. Except for F439, all residues that did not tolerate Trp or other aromatic side-chain perturbation were located on one side of the helical wheel projection (Fig. 2A). While we did not detect any clear periodicity in response to the mutant scan within the lower part of S1 (F439–I446), the effects of Trp mutations in the upper part of S1 (I447–R455) were clearly periodic, supporting a helical character of S1 and indicating that the face containing the affected residues is exposed to one environment more than the other and, presumably, involved in

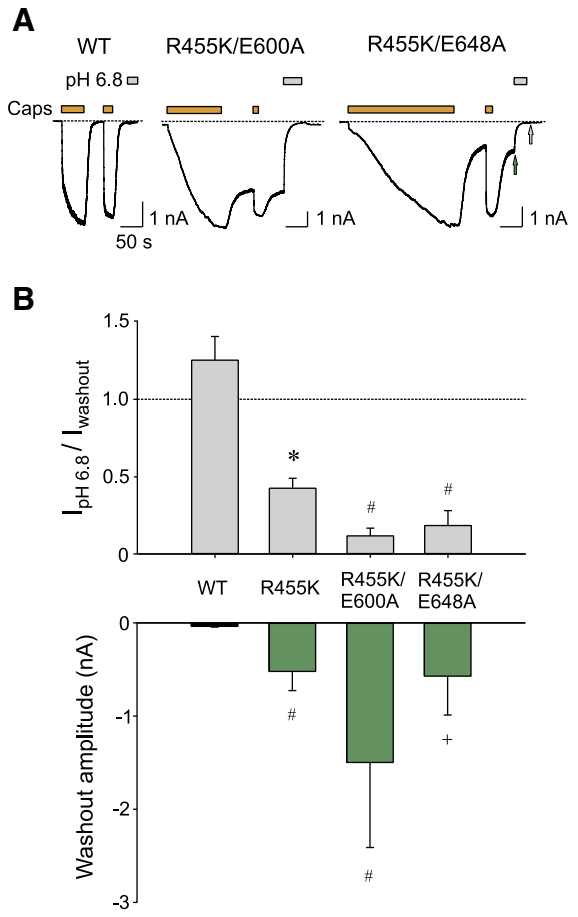

Fig. 6. Acid sensors of TRPV1, E600 and E648, are not involved in proton-induced inhibitory effect. (A) Representative whole-cell current traces taken from HEK293T cells expressing wild-type TRPV1 or double mutants R455K/E600A and R455K/E648A in response to 1 μM capsaicin and pH 6.8. Holding potential −70 mV. (B) Quantification of inward current amplitudes after capsaicin washout (lower plot; time point is indicated by green arrow in panel (A)) and normalized current after application of pH 6.8 extracellular solution (upper plot; grey arrow in panel (A); data were normalized to amplitudes after capsaicin washout). Statistical analysis was performed using the Mann–Whitney *U* test; $^{*}P<0.001$; $\#P<0.01$; $+P<0.05$; $n=3$–19. All data represent mean ± SEM.

tight protein-protein interactions. The current levels through the T449W mutant were small and did not allow for accurate analysis, and the two other Trp mutations, A452W and R455W, located about one helix turn from T449 and from each other, failed to generate functional channels. These residues might form a functionally important contact area interacting with a different part of the TRPV1 protein complex and be involved, together with the residues located in the S4–S5 region R557 and G563, in the same set of gating-related conformational changes. This hypothesis is consistent with several lines of evidence presented here: (i) Experiments examining the responses of the charge-conserving mutation R455K in the extracellular portion of S1 revealed a surprisingly similar phenotype to the gain-of-function mutants R557K and G563S and also to the pore-helix mutant T633A, involved in proton activation. All these mutants did not produce currents in response to low pH 5.5 while pH 6.8 stabilized the closed conformation instead of promoting channel opening as in wild-type TRPV1. (ii) The highly conserved alanine at position 452 did not tolerate any substitution, and its replacement with a small valine side chain could only partially restore the ion channel functionality, indicating that this part of the S1 domain is tightly packed and not exposed to a lipid environment. (iii) Moreover, the neighboring alanine A451, which is less conserved than A452, was intolerant of Trp substitution but was unaffected by serine or valine substitution, indicating the requirement for a small residue at this position and supporting a specific and conserved role for A452. (iv) Our results are in good agreement with the recently proposed topology of the TRPV1 transmembrane region with the mutation-sensitive residues in the predicted buried faces and substitution-insensitive positions in the proposed lipid-exposed faces [33]. Notably, the exception is F439, corresponding to L440 predicted to be exposed to lipids in the human TRPV1 orthologue. This residue is adjacent to the membrane proximal domain of the N-terminus (358–434), a prominent structural component mediating the thermal sensitivity of TRPV1 [28]. This physical proximity suggests that F439 might be important for the correct anchoring of S1 to the plasma membrane and/or downstream gating.

The phenotype of the pore-helix mutant T633A, known to uncouple proton activation, was strikingly similar to the R455K mutant in terms of its capsaicin-, heat-, voltage- and low pH-induced responses (Fig. 4). This construct displayed a slightly increased basal activity, enhanced apparent voltage dependence and impaired heat activation. The kinetic profile of capsaicin responses was slowed compared to the wild-type and the T633A construct was often not able to fully deactivate after capsaicin washout. As in R455K, R557K and G563S; the T633A mutant could not be activated by low pH, on the contrary, protons induced a rapid current deactivation. On the other hand, we confirmed that the second construct known to uncouple proton activation, V538L, was normal in all aspects of TRPV1 functioning except for low-pH-evoked responses [15]. The slowed kinetics of capsaicin-evoked responses in T633A was not observed in the original study by Ryu et al. [15], however, the authors described a similar slowed phenotype for a chimeric TRPV1 channel in which the whole pore helix was replaced with the TRPV2 counterpart. This discrepancy could arise from the fact that the authors used a TRPV1 protein lacking the stretch of 15 residues just before the selectivity filter as a template. This Δ15-TRPV1 was reported to have wild-type responses but it could possibly alter the exhibited phenotype of the T633A mutant.

In the extracellular extent of S1, Trp was not tolerated at position 455 and a positively charged side chain was required to partially preserve TRPV1 channel activity, indicating that this residue participates in an essential electrostatic interaction and that the large guanidinium group of R455 seems to be important for its proper functioning. In our search for possible interaction candidates, we looked at the negatively charged residues most likely to interact with R455 and excluded E458 and E478, located in the same TRPV1 subunit. Based on our functional analysis of the mutant channels and previous studies demonstrating that the pore region of TRPV1 is an active component in the proton sensing and coupling of proton sensor activation [15,20], we propose that it might be the contact surface between S1 and the pore-helix through which protons act to stabilize the closed (resting) conformation of the gain-of-function mutants of TRPV1.

The role of the pore helix in TRPV1 channel gating has been proposed earlier on the basis of finding several gain-of-function mutants with a single amino acid substitution in this region [21]. Using a yeast screening method, Myers and colleagues (2008) identified mutations F640L, T641S and T650S that rendered the TRPV1 channel constitutively active and were hypothesized to mimic the proton-potentiated state of TRPV1, as they were insensitive to the potentiating effect of low pH. Such a simple explanation, however, does not account for all the phenotypic aspects of the overactive mutant channels described in this study. In R455K, R557K, G563S and T633A, we observed markedly slowed kinetics of the onset of the capsaicin-induced responses, which is precisely the opposite of what is expected for TRPV1 channels exposed to the sensitizing effect of low pH [34]. The R455K, T633A and the S4–S5 overactive mutants could not even be activated by severe acidification of the extracellular solution (pH 5.5), in contrast to what has been described for the F640L construct [21]. E600Q is a prototypical mutant that phenocopies the proton-potentiated state of TRPV1. Cells expressing the E600Q mutant channel developed an increased basal current with

repetitive agonist stimulation, similar to what we observed in the mutants described here, but in contrast, the basal current could not be inhibited by protons in E600Q [20]. All these indications point towards the activation mechanism for the R455K, R557K, G563S, and T633A-induced gain-of-function phenotype being more complex and cannot be explained simply in terms of affecting the proton-dependent activation pathway. Moreover, we demonstrate here that the proton-dependent inhibition of the constitutive activating mutation R455K does not depend on E600 and E648. Thus, different mechanisms seem to underlie the constitutive activities produced by mutations at different positions, such as R455K, R557K, G563S, and T633A, where the closed conformation may be destabilized (and stabilized by protons), or with F640L, T641S and T650S, where the open conformation may be stabilized (and insensitive to the potentiating effect of protons).

In the R455K, R557K, G563S and T633A gain-of-function mutants, the elimination of the stimulatory effect of protons could unmask the blocking effect of protons. Indeed, in these mutants, we observed a low-pH-induced diminution of current amplitudes. Most pronouncedly, mildly acidic pH (pH 6.8) was able to reduce the elevated basal current of the mutant ion channels after capsaicin washout by more than 50%, which was accompanied by a decrease in signal noise. This effect was not readily reversible after restoring normal pH, indicating that the ion channels do not simply switch between the protonated and deprotonated conformation. To test the hypothesis that the observed current deactivation is mediated by the same mechanism as the proton-induced decrease in unitary conductance, we wanted to investigate the functional properties of a triple-mutant TRPV1 with a combination of a gain-of-function mutation and the E636Q/D646N double mutation. Unfortunately, E636Q and D646N not only influenced conductance, but also gating properties [19] and their cooperation with the gain-of-function mutations resulted in a severely disrupted TRPV1 phenotype, which we were not able to properly examine.

In summary, the presented data support the possibility that the S1-pore interface might serve to stabilize conformations associated with TRPV1 channel gating and might represent the underlying mechanism involved in the proton-induced inhibition of the constitutive activating mutations in the S1 and S4–S5 region.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by the Czech Science Foundation 305/09/0081, 301/10/1159 and 304/12/G069, the Research Project Fund of the Academy of Sciences of the Czech Republic RVO:67985823 and GAUK 500512.

References

[1] M.J. Caterina, M.A. Schumacher, M. Tominaga, T.A. Rosen, J.D. Levine, D. Julius, The capsaicin receptor: a heat-activated ion channel in the pain pathway, Nature 389 (1997) 816–824.
[2] R. Gaudet, Structural Insights into the Function of TRP Channels, in: W.B. Liedtke, S. Heller (Eds.), TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades, CRC Press, Boca Raton (FL), 2007, (Chapter 25. Available from: http://www.ncbi.nlm.nih.gov/books/NBK5266/).
[3] B. Nilius, K. Talavera, G. Owsianik, J. Prenen, G. Droogmans, T. Voets, Gating of TRP channels: a voltage connection? J. Physiol. 567 (2005) 35–44.
[4] G. Owsianik, D. D'Hoedt, T. Voets, B. Nilius, Structure–function relationship of the TRP channel superfamily, Rev. Physiol. Biochem. Pharmacol. 156 (2006) 61–90.
[5] R. Latorre, C. Zaelzer, S. Brauchi, Structure–functional intimacies of transient receptor potential channels, Q. Rev. Biophys. 42 (2009) 201–246.
[6] S.E. Jordt, D. Julius, Molecular basis for species-specific sensitivity to "hot" chili peppers, Cell 108 (2002) 421–430.
[7] G. Owsianik, K. Talavera, T. Voets, B. Nilius, Permeation and selectivity of TRP channels, Annu. Rev. Physiol. 68 (2006) 685–717.
[8] S.B. Long, E.B. Campbell, R. Mackinnon, Crystal structure of a mammalian voltage-dependent Shaker family K+ channel, Science 309 (2005) 897–903.
[9] S.B. Long, E.B. Campbell, R. Mackinnon, Voltage sensor of Kv1.2: structural basis of electromechanical coupling, Science 309 (2005) 903–908.
[10] S.B. Long, X. Tao, E.B. Campbell, R. MacKinnon, Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment, Nature 450 (2007) 376–382.
[11] S. Brauchi, G. Orta, C. Mascayano, M. Salazar, N. Raddatz, H. Urbina, E. Rosenmann, F. Gonzalez-Nilo, R. Latorre, Dissection of the components for PIP2 activation and thermosensation in TRP channels, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 10246–10251.
[12] H. Salazar, A. Jara-Oseguera, E. Hernandez-Garcia, I. Llorente, O. Arias II, M. Soriano-Garcia, L.D. Islas, T. Rosenbaum, Structural determinants of gating in the TRPV1 channel, Nat. Struct. Mol. Biol. 16 (2009) 704–710.
[13] S. Boukalova, L. Marsakova, J. Teisinger, V. Vlachova, Conserved residues within the putative S4–S5 region serve distinct functions among thermosensitive vanilloid transient receptor potential (TRPV) channels, J. Biol. Chem. 285 (2010) 41455–41462.
[14] S.Y. Lee, A. Banerjee, R. MacKinnon, Two separate interfaces between the voltage sensor and pore are required for the function of voltage-dependent K(+) channels, PLoS Biol. 7 (2009) e47.
[15] S. Ryu, B. Liu, J. Yao, Q. Fu, F. Qin, Uncoupling proton activation of vanilloid receptor TRPV1, J. Neurosci. 27 (2007) 12797–12807.
[16] S. Wang, K. Poon, R.E. Oswald, H.H. Chuang, Distinct modulations of human capsaicin receptor by protons and magnesium through different domains, J. Biol. Chem. 285 (2010) 11547–11556.
[17] I. Dittert, J. Benedikt, L. Vyklicky, K. Zimmermann, P.W. Reeh, V. Vlachova, Improved superfusion technique for rapid cooling or heating of cultured cells under patch-clamp conditions, J. Neurosci. Methods 151 (2006) 178–185.
[18] M.J. Gunthorpe, G.D. Smith, J.B. Davis, A.D. Randall, Characterisation of a human acid-sensing ion channel (hASIC1a) endogenously expressed in HEK293 cells, Pflugers Arch. 442 (2001) 668–674.
[19] B. Liu, J. Yao, Y. Wang, H. Li, F. Qin, Proton inhibition of unitary currents of vanilloid receptors, J. Gen. Physiol. 134 (2009) 243–258.
[20] S.E. Jordt, M. Tominaga, D. Julius, Acid potentiation of the capsaicin receptor determined by a key extracellular site, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 8134–8139.
[21] B.R. Myers, C.J. Bohlen, D. Julius, A yeast genetic screen reveals a critical role for the pore helix domain in TRP channel gating, Neuron 58 (2008) 362–373.
[22] M. Tominaga, M.J. Caterina, A.B. Malmberg, T.A. Rosen, H. Gilbert, K. Skinner, B.E. Raumann, A.I. Basbaum, D. Julius, The cloned capsaicin receptor integrates multiple pain-producing stimuli, Neuron 21 (1998) 531–543.
[23] E. Aneiros, L. Cao, M. Papakosta, E.B. Stevens, S. Phillips, C. Grimm, The biophysical and molecular basis of TRPV1 proton gating, EMBO J. 30 (2011) 994–1002.
[24] M. Li, Y. Yu, J. Yang, Structural biology of TRP channels, Adv. Exp. Med. Biol. 704 (2011) 1–23.
[25] C. Grimm, E. Aneiros, M. de Groot, Dissecting TRPV1: lessons to be learned? Channels (Austin) 5 (2011) 201–204.
[26] J. Grandl, S.E. Kim, V. Uzzell, B. Bursulaya, M. Petrus, M. Bandell, A. Patapoutian, Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain, Nat. Neurosci. 13 (2010) 708–714.
[27] Y. Cui, F. Yang, X. Cao, V. Yarov-Yarovoy, K. Wang, J. Zheng, Selective disruption of high sensitivity heat activation but not capsaicin activation of TRPV1 channels by pore turret mutations, J. Gen. Physiol. 139 (2012) 273–283.
[28] J. Yao, B. Liu, F. Qin, Modular thermal sensors in temperature-gated transient receptor potential (TRP) channels, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 11109–11114.
[29] F. Yang, Y. Cui, K. Wang, J. Zheng, Thermosensitive TRP channel pore turret is part of the temperature activation pathway, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 7083–7088.
[30] J.M. Welch, S.A. Simon, P.H. Reinhart, The activation mechanism of rat vanilloid receptor 1 by capsaicin involves the pore domain and differs from the activation by either acid or heat, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13889–13894.
[31] T.K. Baumann, M.E. Martenson, Extracellular protons both increase the activity and reduce the conductance of capsaicin-gated channels, J. Neurosci. 20 (2000) RC80.
[32] S. Choe, C.F. Stevens, J.M. Sullivan, Three distinct structural environments of a transmembrane domain in the inwardly rectifying potassium channel ROMK1 defined by perturbation, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 12046–12049.
[33] L. Adamian, J. Liang, Prediction of transmembrane helix orientation in polytopic membrane proteins, BMC Struct. Biol. 6 (2006) 13.
[34] T.R. Neelands, M.F. Jarvis, P. Han, C.R. Faltynek, C.S. Surowy, Acidification of rat TRPV1 alters the kinetics of capsaicin responses, Mol. Pain 1 (2005) 28.