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Selective disruption of high sensitivity heat activation but not capsaicin activation of TRPV1 channels by pore turret mutations

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The capsaicin receptor transient receptor potential vanilloid (TRPV)1 is a highly heat-sensitive ion channel. Although chemical activation and heat activation of TRPV1 elicit similar pungent, painful sensation, the molecular mechanism underlying synergistic activation remains mysterious. In particular, where the temperature sensor is located and whether heat and capsaicin share a common activation pathway are debated. To address these fundamental issues, we searched for channel mutations that selectively affected one form of activation. We found that deletion of the first 10 amino acids of the pore turret significantly reduced the heat response amplitude and shifted the heat activation threshold, whereas capsaicin activation remained unchanged. Removing larger portions of the turret disrupted channel function. Introducing an artificial sequence to replace the deleted region restored sensitive capsaicin activation in these nonfunctional channels. The heat activation, however, remained significantly impaired, with the current exhibiting diminishing heat sensitivity to a level indistinguishable from that of a voltage-gated potassium channel, Kv7.4. Our results demonstrate that heat and capsaicin activation of TRPV1 are structurally and mechanistically distinct processes, and the pore turret is an indispensible channel structure involved in the heat activation process but is not part of the capsaicin activation pathway. Synergistic effect of heat and capsaicin on TRPV1 activation may originate from convergence of the two pathways on a common activation gate.

INTRODUCTION

Transient receptor potential vanilloid (TRPV)1 responds to changes in ambient temperature and the presence of capsaicin with exquisite sensitivity (Caterina et al., 1997). Activation of TRPV1 in sensory neurons produces an excitatory transmembrane current that initiates sensory signaling for heat and pain. The high temperature sensitivity of TRPV1 is reflected by a remarkably high Q_{10} value for the activation rate in the range of 20–30 (Clapham, 2003), which is significantly higher than that of "ordinary" ion channels (generally ranged between 2 and 7; DeCoursey and Cherny, 1998) and the temperature dependence of kinetic energy that makes all protein functions intrinsically temperature sensitive (Creighton, 1993). Thermodynamic principles underlying sensitive heat activation have been well studied from the temperature dependence of channel open probability changes derived from current recordings (Liu et al., 2003; Brauchi et al., 2004; Voets et al., 2004; Yang et al., 2010b). Similar to the heat-induced protein-denaturing process, heat activation of TRPV1 and the related heat-sensitive TRPV2–4 channels is associated with a large entropic change that bestows high temperature sensitivity on the channel, as well as a large enthalpic change that matches the entropic change and allows temperature response to occur under physiological temperatures. The balance between entropic change and enthalpic change defines the temperature range in which each TRP channel activates. Although it is generally thought that large entropic and enthalpic changes indicate a substantial conformational rearrangement in the channel protein during heat activation (Clapham and Miller, 2011), what remains unknown is how large entropic and enthalpic changes are determined at the protein-structure level. Indeed, previous studies have suggested several candidate "temperature sensor" structures (Latorre et al., 2009).

In contrast to the uncertainty associated with heat activation, the structural basis for capsaicin activation of TRPV1 is better understood. Capsaicin binds to the protein–aqueous solution interface in the intracellular S2–S4 region of the channel (Jordt and Julius, 2002). High affinity binding is thought to be mediated by π–π interactions between the vanilloid group of capsaicin and the benzene ring of aromatic residues, as seen in the crystal structures of the photosynthetic reaction center of the plant photosystem II (Spyridaki et al., 2000).

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Abbreviations used in this paper: ANK, ankyrin-like repeat; TRPM, transient receptor potential melastatin; TRPV, transient receptor potential vanilloid; WT, wild type.

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Mutations of key aromatic residues in TRPV1 eliminate capsaicin activation (Jordt and Julius, 2002). Additional van der Waals interactions between the hydrophobic tail of capsaicin and the channel protein may also contribute to high binding affinity. As activations of TRPV1 by capsaicin and heat are found to be tightly linked to voltage-dependent activation, it is thought that a common mechanism underlies TRPV1 activation by these three stimuli (Voets et al., 2004). This mechanism is further extended to explain cold activation of transient receptor potential melastatin (TRPM)8 (Voets et al., 2004, 2007). The structural basis for this attractive common mechanism has yet to be identified, whereas outcomes of equilibrium analyses of wild-type (WT) and point-mutant channels start to challenge the applicability of the common gating mechanism to TRPV1 gating (Matta and Ahern, 2007; Grandl et al., 2010; Yang et al., 2010b). An allosteric mechanism developed for TRPM8 (Brauchi et al., 2004) is also considered for TRPV1 (Latorre et al., 2007). Again, structural basis of the allosteric mechanism is not understood. Except for the capsaicin-binding domain, most of the gating modules specified by the allosteric mechanism remain to be assigned to the channel protein structure.

Consistent with the existence of long-range structural couplings, previous investigations have identified many channel structural components for their potential involvement in TRPV1 activation (Latorre et al., 2009). In particular, several recent reports highlight contribution of the extracellular region of the pore domain to channel activation. A screening for gain-of-function mutations identified a residue at the intracellular end of the pore helix, F640, of the rat TRPV1 that is critical for heat activation (Myers et al., 2008). As the mutation F640L promotes both heat and capsaicin activation, similar to mutations in S6 (Susankova et al., 2007), it is possible that this residue directly interacts with the channel's activation gate. In another screening for loss-of-function mutations, residues in the selectivity filter-to-S6 linker region, N652 and Y653, and a residue in the pore helix, N628, were identified to also be involved in heat activation (Grandl et al., 2010). Interestingly, mutations at these sites appeared to inhibit heat activation without a substantial effect on capsaicin activation. Furthermore, proton and other ions also interact with extracellular pore sites in TRPV1 to alter its gating (Jordt et al., 2000; Ahern et al., 2005, 2006). In addition, toxins purified from spider venom are found to stabilize the activated state of TRPV1 by binding to its outer pore region (Bohlen et al., 2010). These findings indicate that the outer pore region has a special contribution to TRPV1 gating.

In a previous study, we found that small fluorescent tags on the pore turret, a 24–amino acid extracellular loop between S5 and the pore helix, reported a substantial movement of this region during heat activation (Yang et al., 2010b). In contrast, similar recordings from the peripheral S1–S4 region did not show any detectable movement. The observation showed that the turret is involved in the conformational rearrangement during TRPV1 activation, suggesting that a better understanding of the role of the turret in TRPV1 gating may help in revealing the heat activation mechanism. How turret movement contributes to heat and capsaicin activation was unclear. To address this important question, in this study we generated systematic turret deletion and replacement mutations, and examined their functional consequences on both heat and capsaicin activation. Our observations confirmed that the turret is not only crucial for supporting the overall structural integrity of the channel pore but also indispensible for highly sensitive heat activation; nonetheless, the turret is not directly involved in capsaicin activation. Our results support an allosteric mechanism by which heat and capsaicin activate TRPV1 through distinct pathways but synergistically promote channel pore opening.

MATERIALS AND METHODS

Molecular biology and cell transfection

The mouse TRPV1 clone was used in this study. Turret deletion and replacement mutants were constructed with overlap PCR. WT cDNA as well as all the mutant cDNAs were fused with the eYFP cDNA at the C terminus to assist identification of the cellular location of expressed proteins. The fluorescence tag did not affect the functional properties of the channel (Cheng et al., 2007). All constructs were confirmed by sequencing. Transfection of HEK293 cells followed standard protocols, as described previously (Cheng et al., 2007). Surface expression and function of each fusion protein were assessed by fluorescence microscopy and patch-clamp recordings 1–2 d after transfection.

Epifluorescence microscopy

HEK293 cells expressing TRPV1 channels were observed under a 60× objective on a microscope (IX-81; Olympus). The filter cube (Chroma Technology Corp.) contained (excitation, dichroic, and emission): HQ500/20, Q515LP, and HQ535/30. Fluorescence images were taken with a camera (HQ CCD; Hamamatsu) driven by MetaMorph software (Molecular Devices). Cellular distribution of expressed channel protein was analyzed using a line-scan function provided by MetaMorph. The fluorescence intensity values along the line were quantified and compared.

Temperature control

Temperature control was achieved by perfusion of preheated or precooled solution, as described previously (Yang et al., 2010b). Solution heating was done with an eight-line heater (SHM-828) driven by a temperature controller (CL-100; both from Harvard Apparatus). A custom-made manifold was attached to the output ports of the heater to deliver solutions to the recording chamber. The manifold provided heat insulation to ensure the heating of cell or membrane patch up to 50°C. Solution cooling was achieved by embedding the perfusion solution reservoir in ice water and delivering the cooled solution through a separate line into the recording chamber. The patch pipette was placed ∼1 mm from the solution output ports. We placed a miniature bead thermistor (TA-29; Harvard Apparatus) 1–2 mm from the pipette tip to ensure accurate monitoring of local temperature. The thermistor's

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temperature readout was fed into an analogue input port of the patch-clamp amplifier (EPC10; HEKA) and recorded simultaneously with current.

Electrophysiology

Patch-clamp recordings were done in the inside-out configuration using an amplifier (EPC10) driven by PatchMaster software (both from HEKA). Additional whole cell recordings were used to test nonfunctional mutants and ensure that we did not miss small currents as a result of low expression. The membrane potential was held at −80 mV, and currents normally were measured at +80 mV. Both pipette solution and bath solution contained 130 mM NaCl, 0.2 mM EDTA, and 3 mM HEPES, pH 7.2. There was no Ca^{2+} in the solution so as to reduce desensitization to capsaicin. To assess the level of leak current, a solution containing 130 mM Ba^{2+} was used to block TRPV1 current in inside-out patches, as described previously (Yang et al., 2010b). Leak current was excluded from current amplitude analyses. For single-channel recordings, two serial Bessel filters were set at 10.0 and 2.9 kHz, yielding an effective filtering frequency of 2.25 kHz. The sampling frequency was 12.5 kHz. The patch membrane was clamped at +80 mV. 10 µM capsaicin was used to maximally activate TRPV1 WT and mutant channels. Single-channel conductance was estimated by constructing all-point histograms and fitting the distribution with a double-Gaussian function, as described previously (Cheng et al., 2007).

Data analysis

To quantitatively assess the temperature threshold for activation, the patch was precooled using a cold solution before recording. A slow increase of temperature was achieved by perfusion of a heated solution. The time course of current activation was recorded. The current–temperature relationship exhibited three major phases. The first slow phase represented mostly temperature-dependent increase in the leak current. It was followed by a rapid takeoff phase that represented heat-induced channel activation. At even higher temperatures, the current declined again as a result of heat-induced inactivation. A linear fit was done for each of the first two phases. The intersect point of the two fitting lines was defined as the activation threshold temperature.

The temperature dependence of the WT and mutant channels was determined in two ways. In the first approach, the channel was first activated by heating to 43°C and then by 10 µM capsaicin at the room temperature. The ratio between current amplitudes measured at these two conditions was calculated as I_heat/I_CAP. When the current started to inactivate before reaching 43°C, I_heat was measured at the peak current. This resulted in an underestimate of the relative amplitude of the heat-induced activation. In the second approach, the slope of heat-dependent current activation was quantified by an R value that was calculated using the equation $R = (I_2/I_1)^{(10/(T2-T1))}$, in which T_1 is the threshold temperature mentioned above, $T_2 = T_1 + 5$°C, and I_1 and I_2 are the current amplitude at T_1 and T_2, respectively. This R value is equivalent to the widely used Q_{10} value measured from the heat-dependent increase in current amplitude, but with one important difference. When Q_{10} was measured from the standard Arrhenius plot, a linear fit to the steepest part of the curve was normally used to estimate the highest Q_{10} value exhibited by the current time course; with our method, the R value was estimated at a fixed 5°C range at the threshold temperature. The R value is expected to be smaller than Q_{10} but would be less sensitive to heat-induced inactivation that was prominent in many turret mutant channels. Because only Q_{10} values calculated from changes in the reaction rate are directly linked to the activation energy as defined by the Arrhenius equation (Hille, 2001), we choose to name our measured quantity differently to avoid confusion.

The dose–response relationship was determined using a solution exchanger (RSC-200; Biologic Science Instruments) with seven separate tubes to deliver different concentrations of capsaicin. The tube number sent by the solution exchanger was fed into an analogue input port of the patch-clamp amplifier (EPC10; HEKA) and recorded simultaneously with current. The stable current amplitude at different concentrations was recorded. A Hill equation was used to fit the dose–response relationship and estimate the EC_{50} value.

All data are shown as mean ± SEM. Statistical significance is determined by the Student's t test and indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Structural modeling of the pore-forming region of mTRPV1

Homology, de novo, and full-atom modeling of the pore-forming domain of mTRPV1 was performed using the Rosetta-Membrane (Yarov-Yarovoy et al., 2006a,b; Barth et al., 2007) and Rosetta symmetry (André et al., 2007) methods. The S5, S6, P-helix, and part of the selectivity region of mTRPV1 were modeled based on corresponding regions in the Kir2.2 channel structure (Tao et al., 2009) using sequence alignment shown in Fig. S1. The loop regions between S5 and P helix (residues I600–S627), P-helix and selectivity filter (residues E637–I643), and selectivity filter and S6 (residues G646–A658) were modeled de novo. 5,000 models were generated in the first round using the cyclic coordinate descent loop modeling (Wang et al., 2007) in the Rosetta-Membrane-Symmetry method, followed by model clustering (Bonneau et al., 2002). The 20 largest cluster models from the first round of modeling were then used as input for the second round of modeling using the kinematic loop modeling protocol (Mandell et al., 2009) in the Rosetta-Membrane-Symmetry method, followed by model clustering. The center of the largest cluster model was also the lowest energy model from the second round of modeling and was chosen as the best model. The structural figure was generated using the UCSF Chimera package (Pettersen et al., 2004).

Online supplemental material

Fig. S1 shows sequence alignment of the pore region among TRPV1s and a number of potassium channels. Fig. S2 shows quantitation of the amplitude ratio between heat- and capsaicin-induced currents for the WT channel and the turret replacement mutants. Figs. S1 and S2 are available at http://www.jgp.org/cgi/content/full/jgp.201110724/DC1.

RESULTS

A turret mutant channel maintains normal capsaicin sensitivity

To investigate the contribution of pore turret to TRPV1 function, we generated a series of turret deletion mutations by deleting the first 10, 17, or all 24 amino acids (named D1, D2, and D3, respectively; Fig. 1 A). We also deleted seven amino acids from the C-terminal end of the turret (named D4). These mutant constructs were tagged with a fluorescent protein at the C terminus and expressed in HEK293 cells. Channel function was assessed by inside-out patch-clamp recording from cells exhibiting strong fluorescence signal (Fig. 1 B). Among them, the D1 mutant channel, which has a relatively small deletion of 10 amino acids from the N-terminal end of the turret, was found to be functional. TRPV1-specific agonist capsaicin elicited robust current responses from both the WT (Fig. 1 C, top) and the D1 channel (Fig. 1 C, bottom). Comparison of the dose–response relationships indicated

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that the capsaicin-induced currents from D1 exhibited near identical ligand sensitivity to that of the WT (Fig. 1 D). The capsaicin EC50 value for the WT channel was estimated to be 0.98 ± 0.05 µM (n = 3), and for D1, the EC50 value was 0.81 ± 0.20 µM (n = 4). These values are not statistically different (P = 0.48).

To assess whether turret deletion affected capsaicin efficacy, we recorded single-channel responses of D1 at +80 mV in the presence of 10 µM capsaicin. To minimize desensitization to the ligand, our recording solution did not contain Ca^{2+}, and we focused on the initial activation induced by capsaicin. Under these conditions, we found that although the WT and the D1 channel differed slightly in single-channel conductance, both channels could reach an open probability near unity at high capsaicin concentrations (Fig. 1 E). As capsaicin exhibited very similar potency and efficacy in activating WT and D1 channels, we conclude that the partial turret deletion in D1 does not directly affect capsaicin activation.

The D1 mutant channel exhibits substantially altered heat response

We next examined heat response of this functional turret mutant. A heat pulse up to 50°C was followed by the application of 10 µM capsaicin to maximally activate the channel, and the amplitudes of the current responses to these stimuli were compared. For the WT channel, the heat-induced current recorded at 43°C was always larger than the capsaicin-induced current (Fig. 2, A, left, and B). In contrast, for D1 the heat-induced current was much smaller than the capsaicin-induced current (Fig. 2 A, right). The reduced heat response was not a result of slow gating kinetics, as lengthening the heat pulse did not yield any increase in the current amplitude (Fig. 2 A, right). Because the capsaicin sensitivities of WT and D1 were very similar (Fig. 1, C–E), the result suggests that heat becomes a less effective activator for the D1 mutant channel (Fig. 2 E).

To further confirm that the heat response of D1 was indeed altered by the turret deletion, we characterized heat response in a way independent of amplitude measurements. To do so, we determined the channel's heat activation threshold as described in Materials and methods. For the WT channel, the threshold temperature was estimated at 37.7 ± 0.3°C (n = 9), and for D1, the activation threshold was shifted significantly lower, to 26.1 ± 0.8°C (n = 7; P < 0.001; Fig. 2, C and D).

A turret partial deletion mutant in the rat TRPV1 was described previously (Ryu et al., 2007; Yao et al., 2010). Like D1, this mutant channel, having a 15–amino acid

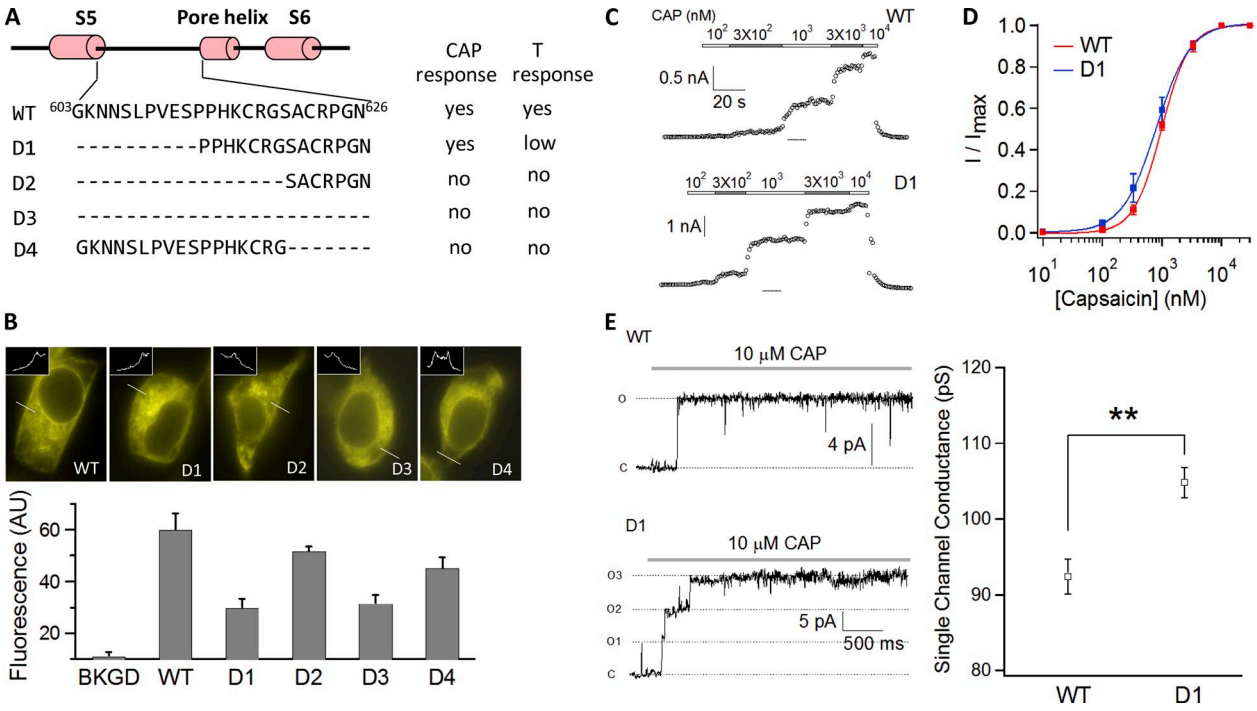

Figure 1. TRPV1 turret deletion mutants. (A) Diagram illustrating turret deletion mutants and their responses to 10 µM capsaicin and heat (50°C or higher). Dashes indicate deletion of amino acids. (B) Cell images demonstrating similar cellular expression patterns between WT TRPV1 and deletion mutants. Fluorescence intensity profile along the indicated line is shown in inset. An intensity peak is visible at the location of plasma membrane. The bottom panel shows quantitation of fluorescence intensity at the plasma membrane. (C) Capsaicin-induced currents from WT (top) and D1 mutant channels (bottom). (D) Dose-dependent activation of WT and D1 by capsaicin. Curves represent fits of a Hill equation to the averaged datasets. The EC_{50} and slope factor values are: 0.98 µM and 1.9 (n = 3) for WT, and 0.80 µM and 1.5 (n = 4) for D1. (E) Single-channel trace (left) and conductance (right) of WT and D1 in response to 10 µM capsaicin. Representative traces of four similar recordings for WT and five for D1. **, P < 0.01.

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deletion, exhibited normal capsaicin response. Heat-induced current was also observed. However, it is not clear from the published report whether heat response of this mutant channel was comparable to that of the WT channel. Nonetheless, a much reduced heat response was observed in a channel having an additional point mutation, T633A (Ryu et al., 2007). The study attributed the functional effect of the point mutation to be mainly on the channel's pH sensitivity. If that is the case, the behavior of T633A would suggest that heat activation of the turret deletion mutant might be already changed (Yang et al., 2010a). Indeed, given that point mutation and chemical modification of single cysteines in the corresponding region of the mouse TRPV1 channel (Yang et al., 2010b) and the neighboring region of the rat TRPV1 channel (Grandl et al., 2010) exhibited substantial gating effects on heat activation, it seems most likely the more dramatic deletion mutation would also affect heat activation. This prediction is supported by additional turret deletion and replacement experiments described below.

Deletions of a larger portion of TRPV1 turret eliminate channel current

We found that, except for D1, most of the turret deletion mutations completely disrupted channel function; no current could be detected from channel-expressing cells challenged by either 10 µM capsaicin or high temperatures up to 50°C (unpublished data; $n = 5–13$). To rule out the possibility that mutant channel proteins failed to traffic to the cell surface, we examined the cellular distribution of the fluorescence signal. As shown in Fig. 1 B, all deletion mutants exhibited a normal cellular distribution similar to that of the WT TRPV1. Scanning of the fluorescence intensity across the cell revealed high protein densities at the plasma membrane (Fig. 1 B, insets), suggesting that mutant channel proteins had trafficked to the plasma membrane.

Collectively, our results from deletion mutants confirmed that a proper turret structure is required for channel function, and a less severe perturbation to the turret substantially and specifically affect heat activation but not capsaicin activation.

Replacing the deleted turret sequences with an artificial sequence restores channel function and normal capsaicin sensitivity

The selective effect of D1 deletion on heat activation suggested that the deletion of other turret regions might have similar gating effects in addition to disruption of pore structural integrity. To investigate this possibility, we sought to recover the function of D2–4 mutant channels by adding back an artificial sequence $(GGGGS)_n$, with the repeat number n being 1 or 3 to roughly match the length of the deleted sequence (Fig. 3 A). The idea was that if the turret deletions altered the overall packing of the channel pore region, reintroducing a flexible "spacer" might restore the proper pore architecture.

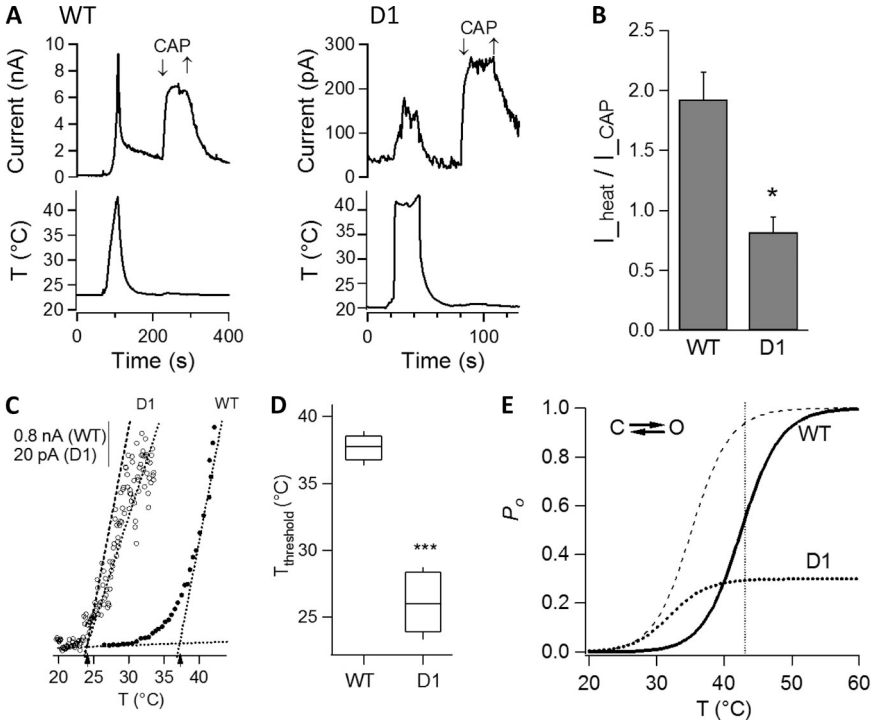

Figure 2. Turret deletion mutant D1 exhibits altered heat response. (A) Example current responses by the WT (left) and D1 (right) to heat and capsaicin. (B) Amplitude ratio between currents induced by heat at 43°C and by capsaicin at 10 µM ($n = 4–5$). *, $P < 0.05$. (C) A left-shift in the activation threshold temperature (arrowheads) is seen in D1 compared with WT. The dash line on the left is of the same slope as the dotted line on the right for WT. (D) Comparison of the activation threshold temperature for WT and D1 channels ($n = 7–9$). For the box-and-whisker plot, the whisker top, box top, line inside the box, box bottom, and whisker bottom represent the 90th, 75th, median, 25th, and 10th percentile value of each pool of measurement, respectively. (E) Hypothetical open probability–temperature relationships for WT and D1 illustrate the observed change in I_heat/I_CAP and the threshold temperature. For WT, values of enthalpic (ΔH) and entropic (ΔS) changes from experimental measurements were used. A reduction of ΔH by 2.3% yielded the dashed curve. A similar reduction of ΔH and a reduction of peak open probability to 30% yielded the dotted curve for D1. A vertical dotted line marks 43°C, at which I_heat was measured.

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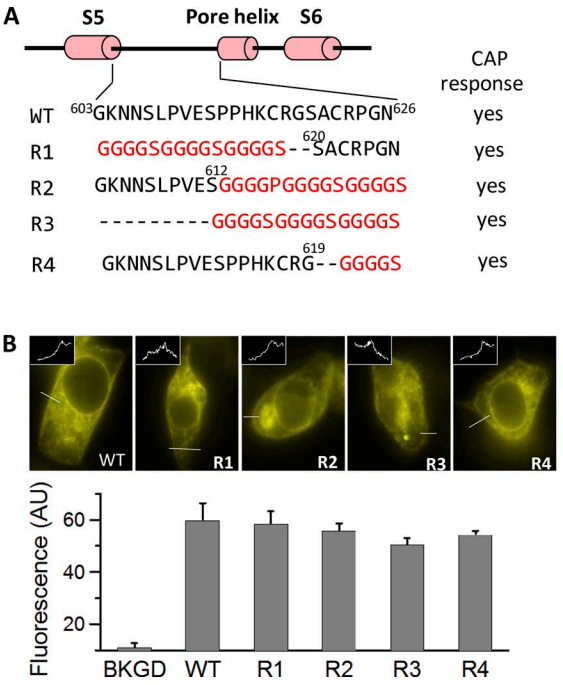

Figure 3. Replacing TRPV1 turret with a polyglycine linker restored capsaicin-induced current. (A) Diagram illustrating the four replacement mutants and their functional evaluation. (B) Cell images demonstrating cellular expression patterns of mutant channels. The bottom panel shows quantitation of fluorescence intensity at the location of plasma membrane. (C) Dose-dependent activation of WT and replacement mutants by capsaicin and fits of a Hill equation. The EC_{50} and slope factor values are: 0.43 µM and 1.2 for R1 ($n = 6$); 0.38 µM and 1.4 for R2 ($n = 3$); 0.55 µM and 1.1 for R3 ($n = 5$); and 1.21 µM and 1.5 for R4 ($n = 4$).

All four mutant channels constructed this way, termed R1 to R4, exhibited normal cellular distribution (Fig. 3 B) and successfully recovered capsaicin response (Fig. 3 C). Importantly, the capsaicin dose–response relationships of all these mutant channels were similar to that of the WT channel, with changes of the EC50 value being less than threefold. The small changes in EC50 reflect a <0.65-kcal/mol energetic effect on the capsaicin activation process by turret replacements. The insensitivity of capsaicin activation to turret mutations exhibited by D1 and R1–4 mutant channels shows that the turret is not directly involved in the ligand-induced activation conformational rearrangement. The result is consistent with our previous observation that capsaicin activation did not substantially affect the turret conformational change (Yang et al., 2010b).

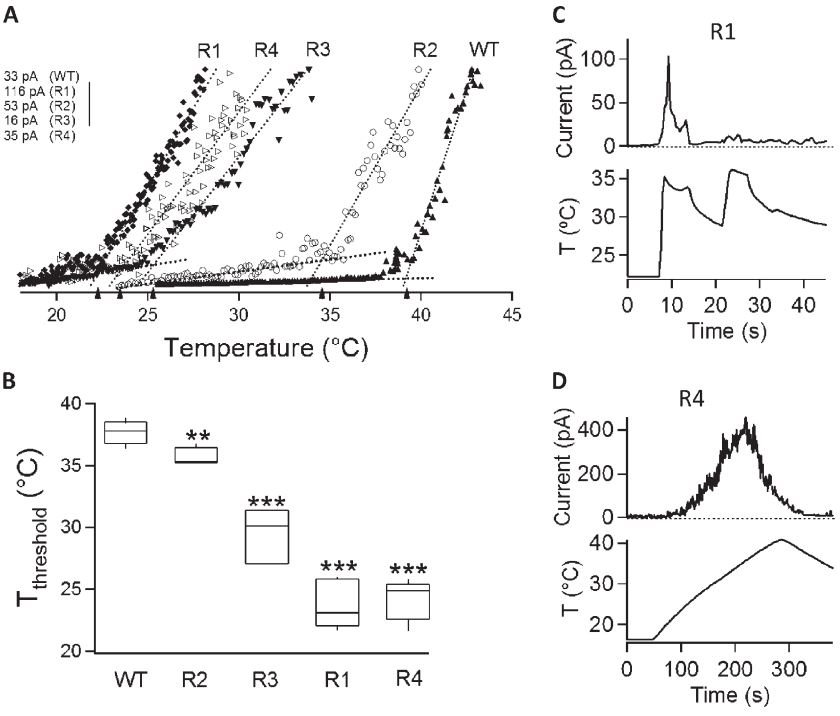

Figure 4. Turret replacement mutants exhibit altered heat response. (A) Example heat-induced currents from the WT and turret replacement mutants. Superimposed dotted lines are fits to the two current phases to determine the activation threshold temperature (arrowheads). (B) Significant shifts in the activation threshold temperature in mutant channels ($n = 5$–9). **, $P < 0.01$; ***, $P < 0.001$. (C) Time-dependent inactivation of the R1 heat response. Recovery from inactivation is extremely slow, so that the second heat pulse failed to elicit a response. (D) Current activation and inactivation of R4 in response to a slow temperature ramp. Note that the channel starts to activate below 25°C and inactivates at a higher temperature before cooling starts.

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Turret replacement mutations affect heat activation

In contrast to the lack of a substantial change in capsaicin sensitivity in the turret replacement mutants, heat response of these channels exhibited large changes. Like D1, these mutants exhibited a significant shift of the heat activation threshold to a lower temperature ($n = 3$–9; $P < 0.01$–0.001; Fig. 4, A and B). In addition, heat-induced currents also appeared to be reduced in amplitude compared with the capsaicin-induced currents (Fig. S2). However, the reduced heat response was difficult to reliably quantify, because heat response from these mutant channels inactivated rapidly and near irreversibly, preventing accurate estimation of the current amplitude. Capsaicin-induced current did not exhibit this type of rapid inactivation. Examples of heat-dependent current inactivation in R1 and R4 are shown at different time scales in Fig. 4 (C and D, respectively). This interesting heat-induced rapid inactivation also reflects changes in the heat activation process in the turret replacement mutants.

Turret replacement mutants lost high temperature sensitivity

One characteristic feature of the highly temperature-sensitive activation of thermoTRP channels including TRPV1 is the steep current–temperature relationship (Clapham and Miller, 2011). We noticed that for the turret replacement mutants, the slope of temperature-dependent current was much reduced. We measured temperature sensitivity by quantifying an R value from the heat-induced current increase of each turret replacement mutant (see Materials and methods), and compared it with the R value of the WT channel. For comparison, we also included in this analysis a voltage-gated potassium channel, Kv7.4, as a representative of the "ordinary" channels that do not exhibit high temperature sensitivity. To avoid the heat- and time-dependent inactivation process described above, the R value was quantified from a 5°C temperature range starting from the activation threshold temperature, as illustrated in Fig. 5 A.

Results from this analysis are summarized in Fig. 5 B. Under our experimental condition (testing voltage at +80 mV in the absence of agonist and Ca^{2+}) and the temperature range, the R value for the WT channel was estimated to be 42.1 ± 10.7 ($n = 7$). All the turret replacement mutants exhibited a reduced R value, with that of R1, R2, and R3 being significantly lower ($P < 0.05$; $n = 5$–7). It was noticed that the reduction in the R value was larger for mutants with a substantial turret replacement (R2 and R3) than those with a smaller replacement (R1 and R4). For R2 and R3, the R values were found to be at the same level as Kv7.4, whose current response to raising temperature presumably reflected the intrinsic temperature dependence of protein functions. In summary, in the turret replacement mutants, the high temperature sensitivity is selectively impaired, suggesting that both the length of the turret and its amino acid contents contribute to the determination of heat response.

DISCUSSION

Polymodal gating is a common feature of many TRP channels. Among them, the polymodality of TRPV1 activation is especially noticeable. At the cellular level, excitation of sensory neurons through activation of TRPV1 by heat and capsaicin is thought to underlie the similar "hot" sensation that these stimuli elicit (Caterina et al., 1997). In the present study, we demonstrated through a series of pore turret deletion and replacement mutations that, at the molecular level, heat activation and capsaicin activation are quite separate processes. The activation

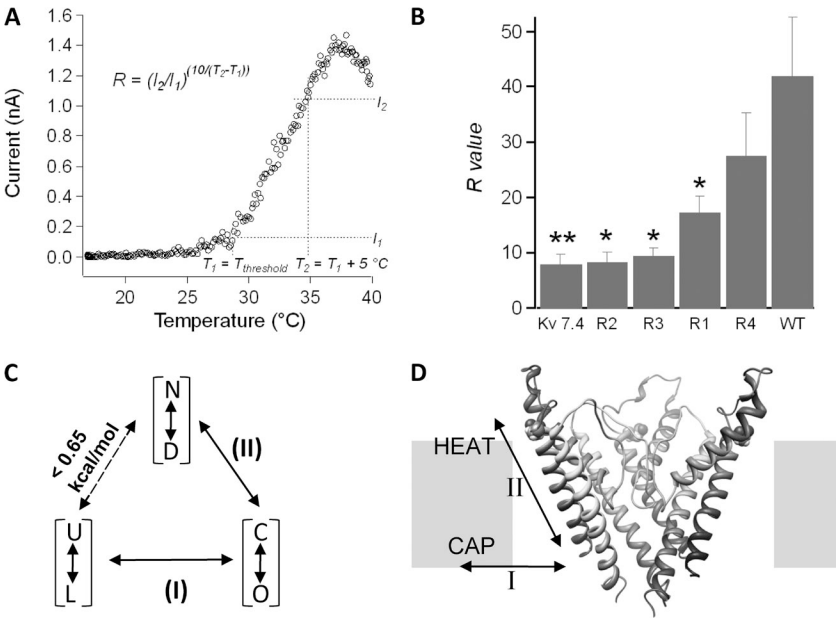

Figure 5. TRPV1 turret replacement mutants lost high temperature sensitivity. (A) Illustration of the R quantification method. Dotted lines indicate the temperature points, T_1 and T_2, and the current levels, I_1 and I_2. (B) Comparison of the R values ($n = 5$–7). *, $P < 0.05$; **, $P < 0.01$. (C) A dual-allosteric coupling model derived for BK channels (Horrigan and Aldrich, 2002) can explain synergistic activation of TRPV1 by heat and capsaicin. Dotted line with arrows indicates a weak coupling. (D) A structural model for TRPV1 activation. The Rosetta-based TRPV1 model contains only the pore-forming S5-to-S6 region. Dashed arrows indicate the capsaicin (I) and heat (II) pathways.

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threshold temperature and the *R* value are two fundamental properties of the heat activation process that specify its dynamic range and sensitivity, respectively. Both properties are strongly affected by structural perturbations to the turret. The finding signifies the contribution of turret to high temperature sensitivity. The rapid inactivation that follows heat activation in the mutant channels likely reflects structural instability of the modified turret, and hence also indicates a structural linkage to the heat-induced process. However, heat activation can be dramatically altered by turret deletion and replacements without substantially affecting capsaicin activation. Similar observations were made from point mutations at outer pore regions (Grandl et al., 2010). Insensitivity of the capsaicin activation process to mutations in the turret and other outer pore regions supports the existence of two separate activation pathways in TRPV1, one mediating agonist activation by capsaicin, and the other mediating heat activation. The existence of two gating pathways that may converge onto the same activation gate is consistent with previous observations (Matta and Ahern, 2007; Grandl et al., 2010; Yang et al., 2010b). By demonstrating that a whole intact turret is required for the highly temperature-sensitive heat activation process, our results also provide new information on the structural basis of the heat activation process.

Many well-studied ion channels exhibit polymodal gating. In this regard, TRPV1 behaves much like the voltage-gated Ca^{2+}-sensitive BK channels, for which both membrane depolarization and the presence of intracellular Ca^{2+} promote channel opening. In a series of detailed studies, Horrigan and Aldrich (1999, 2002; Horrigan et al., 1999) demonstrated convincingly that Ca^{2+} binding and voltage sensor activation occur almost independently but, through their allosteric coupling to the activation gate, exhibit strong synergistic gating effects. A similar dual-allosteric mechanism appears to underlie the gating of TRPV1 by heat and capsaicin (Fig. 5 C). All turret mutations exhibited <0.65-kcal/mol energetic effects on capsaicin activation, which are less than what is expected for direct interactions (Schreiber and Fersht, 1995; Yifrach and MacKinnon, 2002). The result suggests that there is little coupling between the initial capsaicin binding (U↔L) and temperature sensing (N↔D) steps. Instead, capsaicin and heat promote separate transitions that are allosterically coupled to the C↔O transition of the channel pore. Synergy between the two modalities comes from their convergence on the C↔O transition. Although a detailed structural interpretation of this model requires further study on how thermal and chemical energies are transduced along their gating pathways, the physical separation of the two pathways becomes clear (Fig. 5 D). Capsaicin binds to the intracellular binding sites in the S2–S4 region. Conformational change in this region driven by the chemical binding energy is coupled horizontally to the pore. The heat activation pathway is largely unknown, although the outer pore region including the turret and the selectivity filter-to-S6 linker is clearly part of the protein structure participating in the heat-induced conformational rearrangement. It is thus possible that a vertical pathway exists for heat activation.

An important property of allosteric proteins is that a local structural perturbation may exert profound long-range effects through cooperative conformational changes. Just like voltage and Ca^{2+} can strongly influence each other's gating effect on the BK channel through distinct channel structures, capsaicin and heat play a similar tug-of-war on TRPV1 activation through their eventual convergence on the activation gate (Fig. 5, C and D). An implication of this dual-allosteric gating model is that mutations, especially those simultaneously affecting capsaicin, heat, voltage, and other modalities, provide limited indication power in locating the heat sensor. Unfortunately, most of the currently available data are from mutational studies. In this regard, it is important to note that mutations at the turret and some at other outer pore regions (Grandl et al., 2010) exhibit more specific effects on heat activation. It is interesting to find out whether other heat-associated sites may also exhibit a similar behavior.

Distinguishing the activation pathways for heat and capsaicin in TRPV1 not only leads to many potential experimental tests that may help in revealing the gating mechanism of this important cellular sensor, but also it may have important implications for pharmaceutical studies. Indeed, since its discovery, TRPV1 has attracted tremendous interest as an important potential drug target for pain medication. A major hurdle for this effort, however, was revealed when numerous effective TRPV1 inhibitors were found in animal studies and clinical trials to cause acute hyperthermia, reflecting the expected role TRPV1 plays in body temperature homeostasis (Gavva et al., 2008). It was speculated that this issue might be overcome with modality-specific drug candidates (Yang et al., 2010b). Recently, it was reported that the effect on heat activation by several TRPV1 antagonists developed by pharmaceutical companies depends on the pore domain, giving hints that modality-specific regulation of TRPV1 might indeed be possible (Papakosta et al., 2011).

Intensive studies of thermoTRP channels have so far indicated numerous channel regions that contribute to temperature-dependent activation. Exchanging the intracellular C terminus between TRPV1 and the cold-activated TRPM8 channel was found to switch their sensitivity to heat (Brauchi et al., 2006). The result is consistent with an earlier observation from deletion mutations that the last 72 amino acids of the TRPV1 C terminus influenced channel activation (although in a modality-independent manner; Vlachová et al., 2003). In the heat-sensitive snake TRPA1 channel, the large

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ankyrin-like repeat (ANK) domain determines the channel's sensitivity to heat and chemicals (Cordero-Morales et al., 2011). In the related fruit fly heat-sensitive TRPA1 channel, the very N terminus is found to be a regulatory domain of the channel's heat response (Kang et al., 2012), perhaps by directly interacting with the nearby ANK domain. Furthermore, the intracellular segment between ANK and S1 is recently proposed to serve as the thermal sensor for TRPV1 (Yao et al., 2011). Given the limited information from diverse channel types and distinct species, it is difficult to put all studies together and build a consensual picture, especially as results from most studies appear to be exclusive; for example, the latest study by Yao et al. (2011) divided TRPV1 channel protein into several testable regions and ruled out all but one region to be responsible for high heat sensitivity. One potential solution to this dilemma, offered in a recent stimulating article by Clapham and Miller (2011), is that perhaps the high enthalpic and entropic changes associated with heat activation are results of combined contributions from widely distributed sites. A similar idea proposed by Grandl et al. (2010) is that a large Q_{10} value may be the product of multiple smaller Q_{10} values. A physical interpretation of the multiplication of Q_{10} terms is the presence of strong cooperativity among conformational changes. Indeed, the screening of random mutations that affected heat activation in TRPV1 and TRPV3 showed that such point mutations are located throughout these channel proteins (Grandl et al., 2008; Myers et al., 2008). Our previous study (Yang et al., 2010b) and the present study, while highlighting the important role of the pore turret in heat activation, do not distinguish between a single sensor and multiple sensors. Our results, however, argue against the capsaicin-sensing structure being a significant contributor to heat sensing.

How the pore turret participates in heat activation remains to be experimentally determined. Existing evidence from fluorescence recordings suggests that conformational change in the turret during heat activation is quite substantial (Yang et al., 2010b). It raises the possibility that the large entropic and enthalpic changes associated with heat activation might partially arise from the substantial turret conformational rearrangement and the resultant changes in interaction with its surrounding outer pore structures and water molecules. One attractive hypothesis is that the turret structure undergoes heat-induced conformational changes that can be likened mechanistically to heat denaturing. Indeed, protein heat denaturing is a highly temperature-sensitive process. The large enthalpy of the protein denaturing process is a result of cooperative disruption of hydrogen bonds and van der Waals interactions that hold the peptide in its native conformation (Creighton, 1993). Further experiments will be needed to validate this hypothesis; it would be especially interesting to examine whether temperature change can directly induce turret conformational change independent of the rest of the channel protein, as would be required if the turret contributes to heat sensing instead of participating in heat activation in other manners. It should be emphasized, however, that complete denaturing of a part of the channel protein would be unnecessary for high temperature sensitivity, as has been discussed by Clapham and Miller (2011). Complete denaturing of the turret is also not very likely given its importance in supporting the overall pore structure and ion permeation, as demonstrated by the turret deletion mutants in the present study.

Involvement of the outer pore in gating has been found in many channel types (Hille, 2001). In particular, the C-type inactivation of voltage-gated potassium channels is known to involve conformational rearrangements of the outer mouth of the channel pore: closing of the inactivation gate occurs when a part of the selectivity filter-to-S6 linker moves closer to the center of the pore while opening of the gate moves this part away from the central axis (Choi et al., 1991; López-Barneo et al., 1993; Yellen et al., 1994; Baukrowitz and Yellen, 1995, 1996; Liu et al., 1996). Existing evidence suggests that, in both TRPV1 and TRPV3, the selectivity filter-to-S6 linker is involved in heat activation (Jordt et al., 2000; Grandl et al., 2008, 2010), although it remains to be determined how this region moves upon heating. According to our previous fluorescence recordings, the turret appears to move closer to the central axis when TRPV1 activates (Yang et al., 2010b). At the end of the series of conformational changes, the intracellular part of S6 moves away from the central pore axis to open the ion permeation pathway (Salazar et al., 2009).

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