Thermal transient receptor potential (TRP) channels, a group of ion channels from the transient receptor potential family, play important functions in pain and thermal sensation. These channels are directly activated by temperature and possess strong temperature dependence. Furthermore, their temperature sensitivity can be highly dynamic and use-dependent. For example, the vanilloid receptor transient receptor potential 3 (TRPV3), which has been implicated as a warmth detector, becomes responsive to warm temperatures only after intensive stimulation. Upon initial activation, the channel exhibits a high-temperature threshold in the noxious temperature range above 50 °C. This use dependence of heat sensitivity thus provides a mechanism for sensitization of thermal channels. However, how the channels acquire the use dependence remains unknown. Here, by comparative studies of chimeric channels between use-dependent and use-independent homologs, we have determined the molecular basis that underlies the use dependence of temperature sensitivity of TRPV3. Remarkably, the restoration of a single residue that is apparently missing in the use-dependent homologs could largely eliminate the use dependence of heat sensitivity of TRPV3. The location of the region suggests a mechanism of temperature-dependent gating of thermal TRP channels involving an intracellular region assembled around the TRP domain.

**Significance**

Thermal TRP channels are principal molecular entities of transducing thermal and noxious stimuli. The mechanisms by which they detect temperature remain elusive, however. Our and others’ recent studies show that thermal channels, such as the vanilloid receptor transient receptor potential 3 (TRPV3), are strongly use-dependent. Here, by exploring this distinct feature using fast temperature jumps, we have identified a molecular basis for temperature-dependent gating of ion channels.

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Results

Use Dependence of TRPV3. We first examined the wild-type temperature response of TRPV3 and its use dependence evoked by fast temperature jumps. Because the initial activation requires a high temperature $>50^\circ\text{C}$, we have limited temperature pulses to 100 ms so that the channel could be activated in a repetitive manner. Fig. 1A illustrates a train of such heat responses of TRPV3 at 53 $^\circ\text{C}$. The initial current was small, but subsequent stimulations caused progressive increases in responses, which, on average, reached an extent of $>10$-fold after 12–14 repetitions (Fig. 1B).

To see how the sensitization induced by repetitive stimulation affects the temperature sensitivity of TRPV3, we activated the channel by repeated applications of a family of temperature pulses ranging from 30–60 $^\circ\text{C}$ (Fig. 1C). Initially, the channel began to be activated above 50 $^\circ\text{C}$; however, during the repeated run, significant activity occurred at warm temperatures. Fig. 1D compares the current–temperature relationships of the responses between the two runs. Concomitant to the decrease of the activation threshold, the slope sensitivity of the temperature dependence was also drastically reduced. However, the maximum currents between the two runs were similar. Thus, the stimulation mainly altered the steepness of temperature dependence, effectively broadening the responsiveness curve so that the channel became responsive at lower temperatures.

To quantify the change of temperature dependence, we analyzed the van’t Hoff plot of the temperature responsiveness curve (Fig. 1E; applicability of the analysis is discussed by Liu and Qin (14)). The responses of the first run exhibited a linear relationship up to $\sim57^\circ\text{C}$ (black line), with a slope corresponding to an enthalpy change $\Delta H \sim 86$ kcal/mol. The second run displayed a bimodal linear dependence, with a slightly steeper slope at lower temperatures up to $\sim45^\circ\text{C}$, followed by a shallower slope at higher temperatures. Such bimodal temperature dependence could be expected if the gating of the channel became saturating at high temperatures. Thus, we have chosen to fit the lower portion of the curve for the temperature dependence of heat activation, which gave $\Delta H \sim 32$ kcal/mol. Stimulation thus considerably reduced the energetics of gating, indicating that the temperature dependence of TRPV3 is strongly use-dependent.

Membrane Proximal N Terminus Determines Use Dependence. If the temperature dependence of TRPV3 is use-dependent, a perturbation in the structure for temperature sensing would be expected to alter the use dependence. A region that has been identified for temperature sensing by vanilloid receptors is the membrane proximal domain (MPD) on the N terminus, located between the ankyrin repeats and the first transmembrane segment (15). Thus, we investigated whether the same region mediates the use dependence of TRPV3. Because the heat sensitivity of TRPV1 is stable (14), we transferred the MPD of TRPV1 into TRPV3 and evaluated the use dependence of heat sensitivity of the chimeric channel [TRPV3/V1(366–441)], where amino acid numbering refers to mouse TRPV3.

Fig. 2A shows heat responses of the chimera evoked by repetitive temperature jumps at 53 $^\circ\text{C}$. In contrast to wild-type responses, the initial activity of the chimera was large. Furthermore, over a course of $>10$ repetitions, the peak current stayed comparable (Fig. 2B). There was a small increase during the first two or three pulses, but the change was minor, which could have happened due to thermal relaxation of the patch after high-energy exposures. Fig. 2C shows the activation of the chimera with a family of temperature jumps. Here, significant activation was seen at warm temperatures during the first run of stimulation. The time course and temperature dependence of the responses resembled the time course and temperature dependence of the wild-type during repeated activations. Fig. 2D compares the resulting current–temperature relationships between two runs. They were nearly superimposed, indicating that the chimera was stable over repetitive stimulation.

The slope sensitivity of the chimera was estimated with $\Delta H \sim 38$ kcal/mol for both the initial and repeated activations (Fig. 2E). Thus, the same energetic was retained during repeated activations. These energetics were considerably less than the wild-type energetics upon initial activations, but were similar to the energetics during repeated activations, supporting that the chimeric channel indeed resembled the sensitized wild-type channel. The maximum currents were again similar between runs (Fig. 2E). Thus, the overall heat activation profile of the chimera stayed largely unchanged over repetitive stimulation, supporting that the MPD delineates the use dependence of heat sensitivity of TRPV3.

Delineation of Molecular Basis of Use Dependence. We next sought to determine whether the whole membrane-proximal N terminus is required or if a minimal subregion exists to mediate the use dependence of TRPV3. The N-terminal domain we have exchanged is still large, consisting of nearly 80 residues, many of which are not conserved between vanilloid homologs (Fig. 3A).
To narrow down possible effective subregions, we further constructed a series of chimeras between TRPV3 and TRPV1 by exchanging smaller fragments within the domain. Summarized in Fig. 3B are some of the subregions we have successfully exchanged.

We first evaluated the heat activation of the chimeras by repetitive identical temperature pulses (Fig. 3C). When fragments on the N-terminal end of the MPD were exchanged (e.g., residues 365–398), the resulting chimeras still showed use-dependent sensitization (Fig. 3C, Top), which, on average, resulted in greater than sevenfold increases in responses (Fig. 3D, black trace). Next, we extended the exchanged region incrementally toward the C-terminal end of the MPD. When the region between residues 365–414 was exchanged, the chimera exhibited stable responses over repetitive stimulations (Fig. 3C, Middle and D, red). The results thus suggest that the C-terminal end, instead of the N-terminal end, of the MPD is involved in the use dependence of TRPV3. In confirmation, the exchange of fragment 410–414 only recapitulated parent chimeric responses involving the exchange between fragments 365 and 414 (Fig. 3C, Bottom and D).

Fig. 3E and F assessed the stability of temperature dependencies of chimeras using a family of temperature jumps. The large changes with the wild-type channel between two runs of activations persisted in the N-terminal chimera at residues 365–398, but were diminished when the more C-terminal chimera of MPD was exchanged (e.g., residues 365–414, residues 410–414). These C-terminal chimeras displayed fast, low-threshold activations with similar temperature dependencies upon both initial and repeated stimulations.

The van’t Hoff analyses of temperature responsiveness curves confirmed similar energetics between runs for chimeras involving exchanges encompassing the subregion 410–414 (Fig. 3G). These energetics were also similar to the wild-type energetics during

Fig. 2. N-terminal MPD mediates use dependence. (A) Responses of the resulting chimera to repetitive temperature pulses (53 °C). (B) Average plot of fold increase of peak response with respect to repetition of stimulation (n = 11). (C) Responses to a family of temperature jumps in two consecutive runs. (D) Comparison of temperature-dependent responsiveness curves between initial and repeated activations (n = 10). (E) Comparison of energetics (Left, n = 10) and maximum current responses (Right, n = 10). The relative change of the response at 59 °C was plotted. The holding potential was −60 mV.
repeated activations. On the other hand, the residue 365–398 chimera had a large enthalpy upon initial activation ($90 \pm 6$ kcal/mol, $n = 11$), comparable to the wild-type channel. The maximum responses for all chimeras were largely the same between consecutive runs (Fig. 3H). Together, our initial chimeric screening delineated a small, discrete subregion around residues 410–414 for the use dependence of TRPV3.

Loop Region Around Residues 412–414 Is Critical. With position 414 as the C-terminal end, we continued to vary the N-terminal starting position to narrow down the molecular region encoding the use dependence of TRPV3. A minimal region was found to locate around residues 412–414, which corresponds to the loop (residues 404–407) linking two short helices underneath the TRP helix in TRPV1 (Fig. 4A).

Fig. 4B shows that heat responses of the chimera [TRPV3/V1 (412–414)] evoked by repetitive temperature pulses at 53 °C were stable. The responses to a family of temperature jumps also resembled the responses of the parent chimeras (Fig. 4C), yielding overlapping temperature responsiveness curves between consecutive runs (Fig. 4D). The initial activation of the chimera was estimated with $\Delta H \sim 47$ kcal/mol, whereas the repeated activation involved 45 kcal/mol (Fig. 4E). The maximum activity (at 57 °C) was similar between two runs (Fig. 4E). These results indicate that the replacement of the small-loop region between residues 412 and 414 suffices to attain stable heat responses, suggesting that the loop is a major molecular determinant of the use dependence of the heat sensitivity of TRPV3.

A Single-Residue Molecular Switch for Use Dependence. Sequence alignments revealed one notable difference in the loop region (residues 412–414) between different vanilloid receptors: The homologs with use dependence are one residue shorter than homologs with stable heat sensitivity, such as TRPV1 (Fig. 3A). To elucidate the roles of individual residues in the loop, we first examined whether the missing residue (S404 in rat TRPV1) is pertinent to the use dependence of TRPV3. As shown in Fig. 5, the insertion of a serine residue between positions 411 and 412 in TRPV3 resulted in responses with markedly improved stability. The plot of the current–temperature relationship (Fig. 5D) suggests that the second run of activation involved a slightly reduced temperature dependence compared with the first run ($\Delta H \sim 53$ kcal/mol for repeated activations. On the other hand, the residue 365–398 chimera had a large enthalpy upon initial activation ($90 \pm 6$ kcal/mol, $n = 11$), comparable to the wild-type channel. The maximum responses for all chimeras were largely the same between consecutive runs (Fig. 3H). Together, our initial chimeric screening delineated a small, discrete subregion around residues 410–414 for the use dependence of TRPV3.

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the first run and $\Delta H \sim 34$ kcal/mol for the second run; Fig. 5G). These estimates indicate that the mutant is still use-dependent, but to a considerably less degree than the wild-type channel. Thus, the deletion of the serine residue at position 412 in the loop played a major role in inducing the strong use dependence of the wild-type TRPV3.

Other markedly different residues in the loop region between TRPV3 and TRPV1 include N412 and D414, which are E405 and P407, respectively, in TRPV1. To probe the importance of these residues, we substituted them accordingly in wild-type TRPV3, one at a time, for their counterresidues in TRPV1. However, both mutants (N412E and D414P) retained wild-type responsiveness (Fig. 5 E–G). Thus, the mutations at these positions were relatively ineffective in altering the use dependence of the wild-type channel.

**Loop Conformation Controls Heat Sensitivity.** To draw insights into how the loop mediates heat sensitivity and its use dependence in TRPV3, we examined insertions of other residues at position 412 on the loop. The question of particular interest is whether the serine residue as identified above is the best substituent for TRPV3, we examined insertions of other residues at position 412

Among these side chains, the valine residue was found to be the most effective in elimination of use dependence (Fig. 6 A and B), yielding virtually overlapping temperature responsiveness curves between two consecutive runs. The second most effective insertion was threonine (Fig. 6C), for which the responsiveness curves were only slightly shifted between runs. On the other hand, the glycine and alanine insertions were largely ineffective (Fig. 6 D and E), albeit alanine slightly reduced the $\Delta H$ of initial activation (Fig. 6G), whereas the insertion of an asparagine residue, which is polar and also larger in size, showed an intermediate effect (Fig. 6 E and H).

Overall, the data support that heat activation of TRPV3 strongly depends on the side-chain properties of residues in the loop, particularly at position 412. The ineffectiveness of the glycine insertion implies that the loop flexibility is not a critical determinant, whereas the effectiveness of valine rules out the significance of polarity but favors a role for the size of the side chain. The order of the relative effectiveness of insertions between G, A, and S is also consistent with an effect of the side-chain size. The optimal size appears to be achieved with valine or threonine with branched chains. The results thus support that the conformation of the loop, which is dependent on side-chain sizes, is important for heat sensitivity and its use dependence of TRPV3.

**Discussion**

By application of rapid temperature jumps, we have resolved a more complex temperature activation profile of the vanilloid receptor TRPV3 than commonly thought as a warmth detector (4–6). The channel possesses a high noxious activation threshold $>$50 °C, similar to TRPV2 (3), upon initial activation. It is only after intense stimulation that it becomes responsive to warm temperatures, TRPV3 thus can function as either a nociceptor or a warmth receptor. The change of the activation threshold of the channel does not occur by a simple shift of the gating curve, as usually occurs for sensitization of a channel due to messenger-based regulations. Instead, it results from the change of the steepness of the gating curve. The use-dependent sensitization thus occurs at the expense of a decreased discriminative power over small temperature gradients.

The sharp steepness of temperature dependence of thermal channels is generally associated with the large energetic involved in temperature sensing by the channels. Thus, a straightforward interpretation of the large change in the slope sensitivity of TRPV3 is that the process of temperature sensing by the channel becomes altered during activation. For more complex gating mechanisms such as an allosteric paradigm (16), where the stimulus sensor domain is allosterically coupled to the gate, the slope of the gating curve may depend on both stimulus sensor properties and strength of allosteric coupling. However, as illustrated by simulations in Supporting Information, a change in the coupling strength in this case predicted opposite effects on activation threshold and slope sensitivity. By fitting explicitly with an allosteric scheme (Supporting Information), we found that such a model was difficult to reconcile with the responsiveness curves of TRPV3 both before and after sensitization if temperature sensor properties were unchanged. These analyses suggest that a change in allosteric coupling alone could not account for the type of simultaneous reductions of the activation threshold and slope sensitivity of TRPV3, and thus still support changes in intrinsic temperature dependence of the channel. Consistently, the loop region underlying the use dependence of TRPV3 is a part of the N-terminal MPD. Among the various molecular regions that have been found to affect activations of thermal channels (15, 17–21), the MPD has been implicated for temperature sensing by vanilloid receptors (15). Thus, the location of the loop also supports altered temperature sensing underlying the use dependence of TRPV3.

The new structures of TRPV1 reveal several intriguing features in the identified loop region which may be pertinent to the use dependence of TRPV3 (22; Supporting Information). The critical serine at position 404 is in proximity to the S2–S3 linker, with distances to V508 (closed: 4.48 Å, open: 3.93 Å) and D509 (closed: 4.73 Å, open: 4.4 Å) in the range for van der Waals interactions. Two other residues on the loop, T406 and P407, are H-bonded to ankyrin repeats via residue S342 in the closed state and residue G344 in the open state, respectively. Thus, the loop interfaces with the S2–S3 linker on the top and the ankyrin repeats on the bottom. This arrangement of the loop and its dynamics suggest a possible “click-and-hold” model for activation...
of TRPV3. Because the serine residue is missing in TRPV3, the loop may become dislocated from the S2–S3 linker. Then, the recovery of S404 in TRPV3 mutants presumably plays a role in restoring the interactions with the S2–S3 linker. The change of the loop position can incur further changes in local structures, such as the MPD and adjacent ankyrin repeats, with a functional impact on heat sensitivity and stability. On the other hand, the comparison of the closed and open structures of TRPV1 indicates that the distances between S404 and the S2–S3 linker become shorter in the open state than in the closed state, suggesting a trend that the loop moves toward the S2–S3 linker during channel opening. In the wild-type TRPV3, such a movement upon initial activation may bring the loop sufficiently close to the S2–S3 linker to forge new interactions between them, leading the channel to adopt a structure subsequently similar to the serine insertion mutant. The appearance of this new structure would underlie the hysteresis of the wild-type TRPV3. According to our energetic analyses, the opening from the new structure, which corresponds to the activation during repeated stimulation, involved \( \Delta H \sim 30 \text{ kcal/mol} \), compared with \( \Delta H \sim 90 \text{ kcal/mol} \) for the initial activation. This change places an estimate of an energetic change of \( \Delta H \sim 60 \text{ kcal/mol} \) between the initial and new structures. In summary, the structural and functional data both support that the positioning of the loop has influences on structures of TRPV3, especially the elements for temperature sensing, and predict its dynamic interactions with the S2–S3 linker as a mechanism for the use dependence of the channel.

**Materials and Methods**

**Temperature Jumps.** Temperature jumps were produced by laser irradiation (23). Constant temperature steps were generated using the current of an electrode for feedback control. Temperature was calibrated offline from the electrode current based on temperature coefficient \( (Q_1)_T \) of electrolyte conductivity.

**Other Materials and Methods.** Details for the method described above and for cell culture, expression, electrophysiology, and data analysis are provided in Supporting Information.

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