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Use Dependence of Heat Sensitivity of Vanilloid Receptor TRPV2

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ABSTRACT Thermal TRP channels mediate temperature transduction and pain sensation. The vanilloid receptor TRPV2 is involved in detection of noxious heat in a subpopulation of high-threshold nociceptors. It also plays a critical role in development of thermal hyperalgesia, but the underlying mechanism remains uncertain. Here we analyze the heat sensitivity of the TRPV2 channel. Heat activation of the channel exhibits strong use dependence. Prior heat activation can profoundly alter its subsequent temperature responsiveness, causing decreases in both temperature activation threshold and slope sensitivity of temperature dependence while accelerating activation time courses. Notably, heat and agonist activations differ in cross use-dependence. Prior heat stimulation can dramatically sensitize agonist responses, but not conversely. Quantitative analyses indicate that the use dependence in heat sensitivity is pertinent to the process of temperature sensing by the channel. The use dependence of TRPV2 reveals that the channel can have a dynamic temperature sensitivity. The temperature sensing structures within the channel have multiple conformations and the temperature activation pathway is separate from the agonist activation pathway. Physiologically, the use dependence of TRPV2 confers nociceptors with a hypersensitivity to heat and thus provides a mechanism for peripheral thermal hyperalgesia.

INTRODUCTION

Pain-producing stimuli are detected by specialized sensory neurons known as nociceptors in the peripheral nervous system. Noxious heat excites two types of primary afferents, the A fibers and C fibers whose nerve terminals innervate the skin. Two transduction mechanisms have been distinguished for noxious heat detection (1). One is characterized with a low temperature threshold (~45°C) and is mediated by two groups of heat-sensitive primary afferents, namely, the mechano-heat sensitive C fibers (CMH) and the mechano-heat sensitive type II Aδ-fibers (AMH II). The other involves a group of high-threshold nociceptors, namely, the type I mechano-heat sensitive Aδ-fibers (AMH I), which exhibit a temperature threshold >52°C. The temperature thresholds of these transduction mechanisms closely match those of the vanilloid receptors TRPV1 and TRPV2, respectively (2,3). Thus the vanilloid receptor TRPV1 has been suggested to subserve heat transduction in the low-threshold heat-sensitive nociceptors (4–8), while the vanilloid receptor TRPV2 is a candidate of the heat transducer in the high-threshold AMH I afferents (9–11).

The vanilloid receptors like TRPV1 and TRPV2 are members of a group of transient receptor potential (TRP) channels. These so-called thermal TRP channels are directly activated by temperature (12). Different thermal channels have different temperature thresholds, and as thermal transducers, they all possess unprecedented temperature dependence, which allows them to discriminate a subdegree temperature gradient. However, how thermal TRP channels achieve their unique biophysical properties remains elusive. One outstanding issue has been how temperature is sensed by the channels (13). As a nonspecific stimulus, temperature can influence every part of a protein. Thus the activation of thermal TRP channels by temperature could conceivably result from a global effect of thermal stress over entire proteins. Alternatively, the thermal energy that confers channel activation may be confined within the channel structure, that is, the channel contains specialized molecular domains that act as a temperature sensor to allow for sensitive detection of temperature by the channel.

Despite fundamental differences between the two candidate mechanisms, the existing studies have led to evidences that seemingly support both propositions. Thermal TRP channels respond to both physical and chemical stimuli. Biophysical modeling of this polymodal responsiveness supports a mechanism by which the gating by temperature

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involves modular thermal sensors (14–16). However, simple two-state models that are consistent with a global temperature effect have also been proposed to describe the activation of thermal channels by temperature (17). Chimeric studies concerning the molecular basis of temperature sensitivity of thermal channels have led to identification of several regions including, for example, the N-terminus (18–21), the C-terminus (22), and the pore domain (23–25), among others (26,27). Thus the regions pertaining to temperature sensitivity of thermal TRP channels are seemingly delocalized and spread throughout the whole channel. On the other hand, in the only study where chimeric changes extend to whole channels, only an N-terminal domain is found to predominate in the energetics of temperature activation in vanilloid receptors, while other domains including the C-terminus and transmembrane regions have minor contributions (18). One possible explanation for these seemingly discrepant findings is that temperature activation of thermal channels may comprise multiple steps, as is often the case for allosteric proteins. The different regions that have been identified, although diverse in their locations, may mediate distinct activation steps, such as temperature sensing, pore opening, and allosteric coupling between them.

To draw further insights upon the underlying mechanisms of thermal channels, we here provide an in-depth characterization of the heat sensitivity of the vanilloid receptor TRPV2. The gating of the channel is strongly use-dependent, and further biophysical analyses of the use dependence suggest that it is associated with changes in properties of temperature sensing. As a result of the use dependence, the heat activation of the channel can change from being strongly temperature-dependent to only nominally temperature-dependent, and the activation threshold decreased from high noxious temperatures $>50^{\circ}$C to warm temperatures. Importantly, the changes in temperature dependence are only evoked by heat but not agonist stimulation, although the agonist activation itself also exhibits use dependence. These results support that the structural basis for temperature sensing in TRPV2 is highly flexible and the activation pathway evoked by temperature is separate from that by agonist, both of which are consistent with a confined thermal sensitivity within the channels.

MATERIALS AND METHODS

Cell culture and expression

HEK 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% penicillin/streptomycin, and were incubated at 37°C in a humidified incubator gassed with 5% CO_2. Transfection was made at a confluence of ~80% by calcium phosphate precipitation. Monomeric red fluorescent protein was cotransfected for laser beam positioning. Experiments took place usually 10–28 h after transfection. The rat rTRPV1 and rTRPV2 clones were provided by David Julius (University of California-San Francisco, San Francisco, CA).

Electrophysiology

Patch-clamp recording was made in whole-cell and excised-patch configurations. Currents were amplified using an Axopatch 200B amplifier (Axon Instruments, Jakarta, Selatan, Indonesia), low-pass filtered at 5–10 kHz through the built-in eight-pole Bessel filter, and sampled at 10–20 kHz with a multifunctional data acquisition card (National Instruments, Austin, TX). Data acquisition was controlled by a home-made program capable for synchronous I/O and simultaneous control of laser and patch-clamp amplifier. Patch pipettes were fabricated from borosilicate glass capillary (Sutter Instrument, Novato, CA) and fire-polished to a resistance of <5 MΩ when filled with 150 mM CsCl solution. Pipette series resistance and capacitance were compensated using the built-in circuitry of the amplifier (50–70%), and the liquid junction potential between the pipette and bath solutions was zeroed before seal formation. Currents were evoked from a holding potential of -60 mV.

Bath solutions for whole cell recording consisted of 150 mM NaCl, 5 mM EGTA, and 10 mM HEPES, pH 7.4 (adjusted with NaOH). Electrodes were filled with 140 mM CsCl, 10 mM HEPES, and 1 mM EGTA, pH 7.4 (adjusted with CsOH). Symmetric solutions of 150 NaCl were used for excised patch recording. The pH of the HEPES-buffered solutions changed by ≤ 0.4 unit over 22–55°C. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Fast temperature jump

Temperature jumps were produced by laser irradiation using a single emitter high-power infrared laser diode, as previously described in Yao et al. (28). In brief, the diode was mounted on a cooling block and operated at room temperature. Laser emission from the laser diode was launched into a multimode fiber with a 100-μm core diameter and 0.2 NA. The other end of the fiber was positioned close to cells as the perfusion pipette normally was. The laser diode was driven by a pulsed quasi-CW current power supply (Lumina Power, Bradford, MA). Pulsing of the controller was controlled by computer through the data acquisition card using a custom program. A green laser line (532 nm) was coupled to the fiber to aid alignment. The beam spot on the coverslip was identified by illumination of monomeric red fluorescent protein-expressing cells using the green laser.

Constant temperature steps were generated by irradiating the tip of an open pipette and using the current of the electrode as the readout for feedback control. The laser was first powered on for a brief duration (<0.75 ms) to reach the target temperature and subsequently modulated to maintain a constant pipette current. The modulation pulses were stored and subsequently played back to apply temperature jumps to whole cells or membrane patches. Between consecutive temperature pulses, laser power was adjusted manually and the adjustment generally took less than half a minute. Temperature was calibrated offline from the pipette current based on the temperature dependence of the electrolyte conductivity.

RESULTS

Use dependence of heat activation of TRPV2

The vanilloid receptor TRPV2 has a high temperature activation threshold at ~52°C. This temperature responsiveness range is difficult to access by conventional temperature controls. As a result, the heat sensitivity of the channel has been less studied than its homologs with lower activation thresholds. To cope with the problem, we used a fast temperature jump system capable for delivering a short temperature pulse to minimize heat stress on patches. With such

stimulation, we were able to record TRPV2 currents in its responsiveness range between 50 and 60°C while maintaining patch stability.

Fig. 1 *A* illustrates current responses of TRPV2 evoked by fast temperature jumps in HEK 293 cells. Significant currents were activated between 50 and 53°C, consistent with the previously reported activation threshold at ~52°C. Once above the threshold, the current was increased rapidly over only a few degrees of temperature changes (~53–56°C). A notable feature of the response is that its time course was very slow, following a nearly linear increase with time.

In patches that remained stable after exposure to the first run of fast temperature jumps (to improve the success rate of the second run, we had usually limited the maximum temperature jump to 55–56°C during the first run), we attempted to activate the channel again with the same temperature jumps. These same stimuli, however, resulted in different responses. Fig. 1 *B* shows the heat-activated current over the second run of stimulation, recorded from the same cell as in Fig. 1 *A*. The responses exhibited several characteristic changes. First, the threshold of temperature activation became significantly lower. A detectable activity began to occur in the warm temperature range (31–40°C). Second, the time course of the activation became considerably faster. While the current rose almost linearly with time over the initial stimulation, the repeated activation tended to have an exponential time course. Third, the temperature dependence of the channel was profoundly changed. The large increment of the heat response occurring between 53 and 56°C during the first stimulation no longer occurred during the repeated stimulation.

Fig. 1 *C* summarizes the temperature dependence of the peak current response measured at the end of each temperature pulse. As expected, the activation of the channel during the initial run exhibited a steep slope sensitivity above 50°C. Below 50°C, the current was increased slowly, suggesting that it could be attributed to background sources such as leakage currents. Compared to the first-run response, the temperature response obtained in the second run exhibits a much shallower slope, which spans a broad temperature range from 31 to 56°C.

Fig. 1 *D* compares the peak current at ~56°C between the first and second runs. The change of the peak current was relatively small. Thus, the responses differ mainly in the temperature activation threshold and the slope sensitivity after the initial stimulation. These results indicate that the heat activation of TRPV2 is strongly use-dependent. The use dependence effectively sensitizes the channel so that warm temperatures suffice to activate it after initial activation.

Independence on cellular regulation

To see if the use-dependent change in heat activation of TRPV2 depends on intracellular modulation of the channel, we repeated the above experiment in excised membrane patches. Fig. 2, *A* and *B*, shows representative currents of TRPV2 recorded from an inside-out patch.

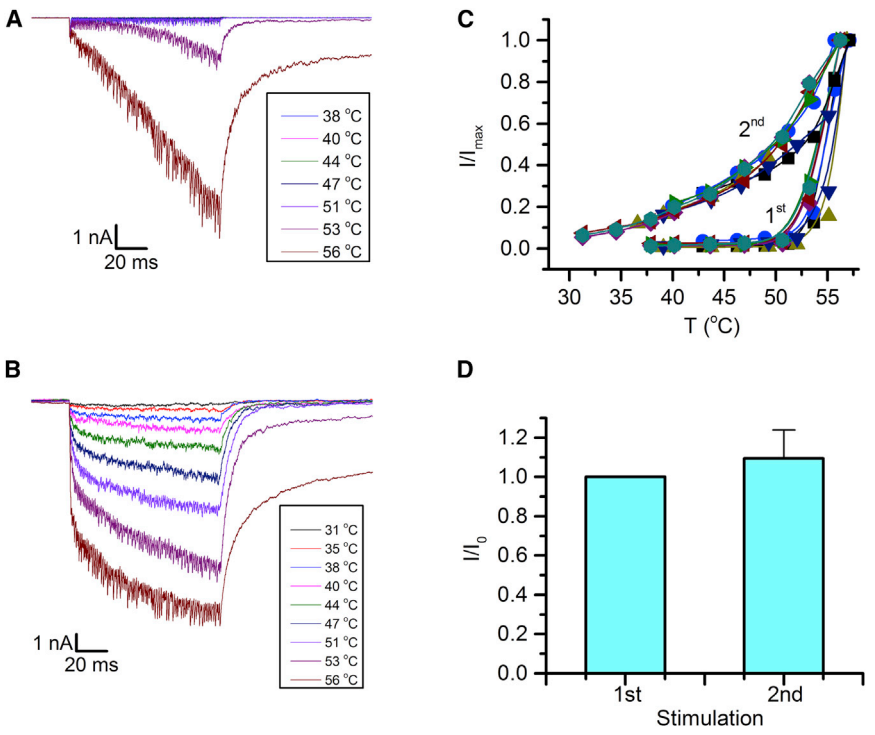

FIGURE 1 Use dependence of heat activation of TRPV2. (*A*) Heat response of TRPV2 evoked by fast temperature jumps between 38 and 56°C. Each temperature pulse lasted for 100 ms with a rise time <0.75 ms. (*B*) Recordings from the same cell by repeated application of the same temperature jumps. (*C*) Current-temperature relationships for heat responses over the first and second run of stimulations. Peak responses at the end of temperature pulses were measured and normalized to the maximal response at ~56°C. (*D*) Comparison of the maximum response between the first and second run of activations ($n = 7$). Recordings from HEK 293 cells transiently transfected with rat TRPV2. Holding potential at −60 mV. To see this figure in color, go online.

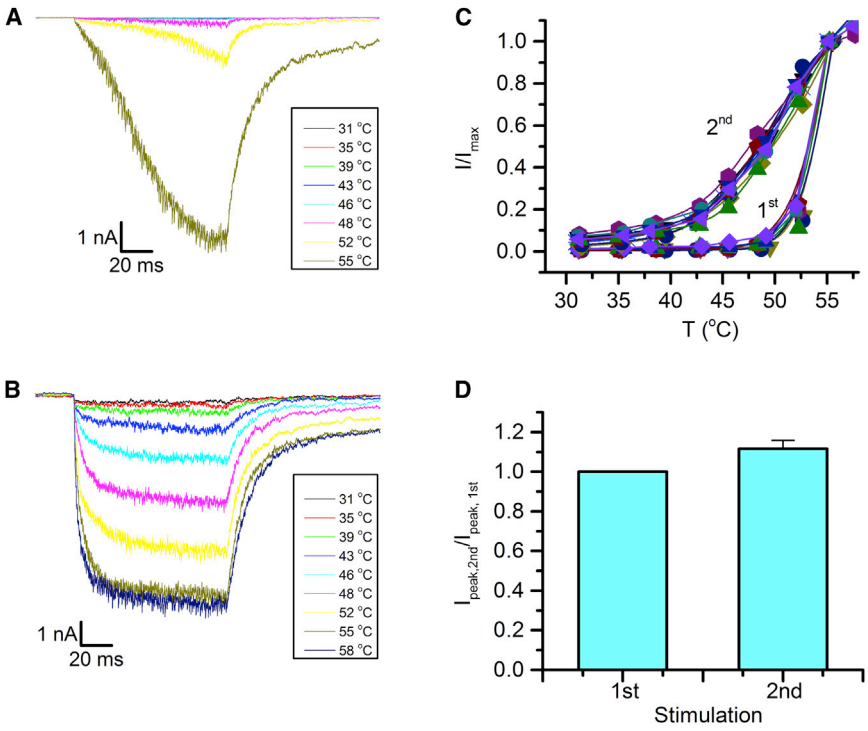

FIGURE 2 (*A–D*) Parallel recordings of heat responses of TRPV2 from inside-out membrane patches. Stimulation protocols and recording conditions were similar to those in Fig. 1 except for plots of current-temperature relationships where currents were normalized to responses at ~55°C. To see this figure in color, go online.

Heat remained to activate the channel in cell-free conditions. The response also changed drastically between initial and repeated stimulation. Upon initial stimulation, a significant current was activated at temperatures between 48 and 52°C. The activation had a slow time course, and the current was extremely sensitive to small temperature elevations. When the same patch was stimulated again, however, the channel became responsive at much lower temperatures (31–55°C; Fig. 2 *B*). The time course of the activation was also more rapid, while the current amplitude tended to increase more uniformly with temperature changes. These characteristic changes were parallel to those of whole-cell responses. The current-temperature relationship in Fig. 2 *C* confirms that the initial stimulation of the channel caused profound decreases in subsequent temperature dependence of the channel, although the peak response as measured around 55°C remained relatively similar (Fig. 2 *D*). Thus the channel exhibited similar use dependence in cell-free membrane patches, supporting that the use dependence of its heat sensitivity is membrane-delimited.

One difference noticed between whole-cell and patch configurations was that the heat response in cell-free membrane patches tended to run down at extreme temperatures (>57°C) while the whole-cell current was usually stable in the similar temperature range. This apparent rundown could be due to changes in membrane's physical properties between the two configurations, causing channels in patch configurations that were less tolerant to heat stresses than in the whole-cell configuration. Because it occasionally occurred at very high temperatures, the rundown did not obscure the detection of use dependence of the channel.

Independence on stimulation protocol

To better understand the use dependence of TRPV2, we also tested it with a simplified stimulation protocol using identical temperature pulses. Fig. 3 *A* shows representative heat responses to repetition of same-temperature jumps at 53°C. The current was increased progressively with repetition of stimulation. On average, the final response was ~5-fold the initial one. Thus the sensitization of TRPV2 was also attainable with repetition of same-temperature jumps. In addition to the current amplitude, the kinetics of the response was also altered. When first stimulated, the channel showed a small response rising slowly (the rapid drop at the beginning of the pulse was due to leakage current). After sensitization, a large current was activated rapidly after temperature jump. Thus, the sensitization of the channel is accompanied with changes in both amplitude and kinetics of responses, which are consistent with changes observed with stimulation by multiple temperature pulses.

We also examined whether the interpulse duration plays a role in the sensitization of the channel. Fig. 3 *C* shows the experimental protocol where the channel was first activated and sensitized by two identical pulses at 53°C, followed by a maximum stimulation pulse at 56°C (to obtain the maximum response from the patch). The duration between the two 53°C pulses was varied (1 vs. 5 min). Fig. 3 *D* compares the sensitization of the response at 53°C, measured as the peak response at the end of the second pulse relative to

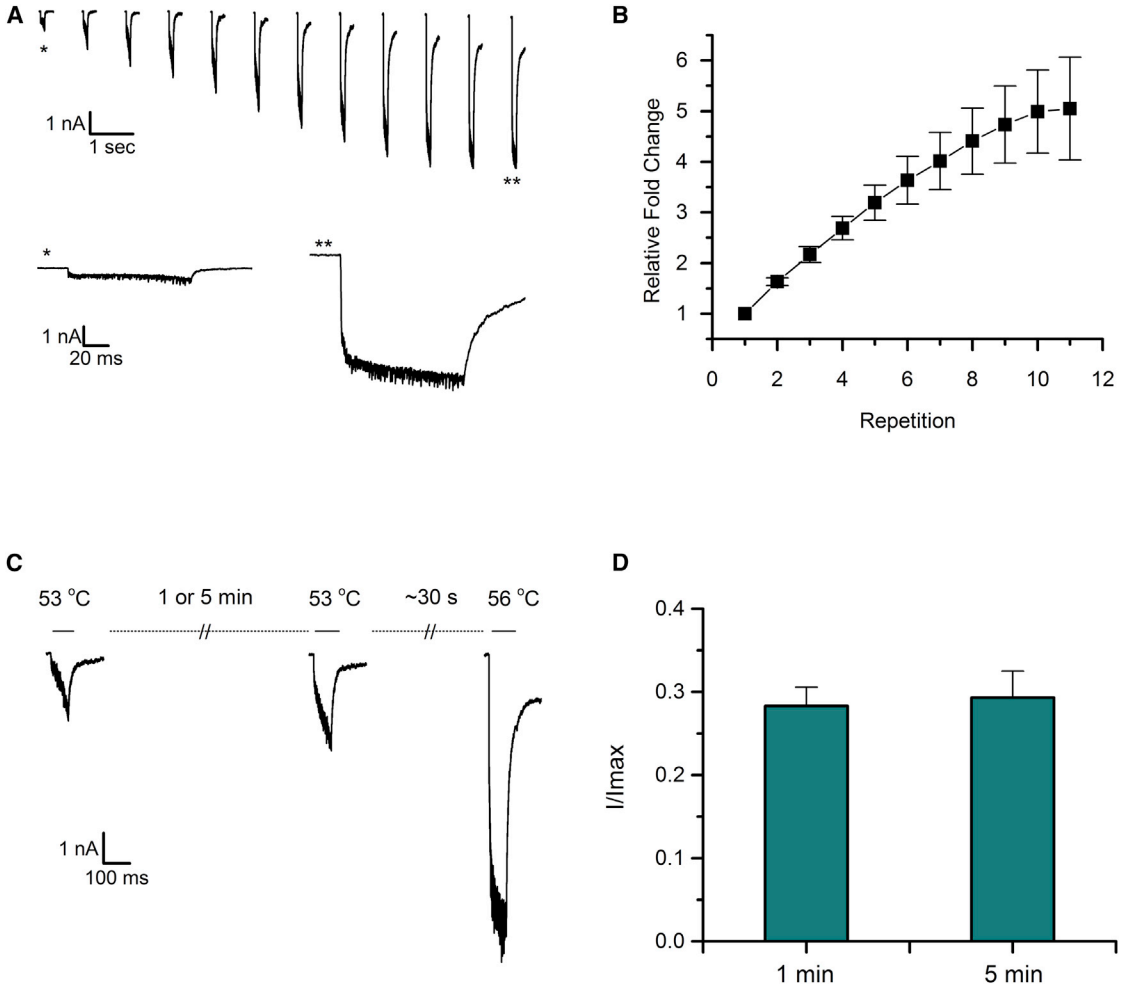

FIGURE 3 Repetitive heat activation of TRPV2. (*A*) Heat responses of TRPV2 evoked by the same temperature pulse (53°C) applied repeatedly (*top*). The inset traces (*bottom*) show an expanded view of the first and last responses (marked by * and **, respectively). (*B*) Statistical plot showing the increase of peak response (measured at the end of temperature pulse) with the repetition of stimulation ($n = 10$). (*C*) Recordings for testing effects of interpulse durations. The channel was first activated and sensitized by applications of two identical temperature pulses at 53°C, followed by a third pulse at 56°C to obtain the maximum activity in the patch. The duration between the two sensitizing pulses was either 1 or 5 min. (*D*) Comparison of sensitization with different interpulse durations. The sensitized peak response at 53°C during the second pulse relative to the maximum current in the same patch is shown ($n = 10$). Recordings from HEK 293 cells transiently transfected with rat TRPV2. Holding potential at −60 mV. To see this figure in color, go online.

the maximum current at 56°C from the same patch. The responses were similar within error limits irrespective of interpulse intervals, suggesting that the sensitization of the heat response is dependent on heat activation of the channel.

Irreversibility of use dependence

We found that the use-dependent changes of TRPV2 generally lasted throughout experiments. For a more quantitative analysis, we examined the recovery of TRPV2 using a paired-pulse test (Fig. 4), where the heat response of the channel to a test temperature pulse (53°C) was measured before and after a conditioning pulse (56°C). The duration between the conditioning pulse and the second test pulse was varied to assess the recovery of the channel. In the first experiment, we applied the second test pulse immediately after the conditioning pulse (the time interval was limited by the time taken for adjusting laser intensity and was on the order of half a minute). In the other test, we increased this time interval to ~30 min. In both cases, the heat response evoked by the second test pulse differed considerably from the first test response (Fig. 4 *A* versus Fig. 4 *C*). The peak current was significantly increased while the time course of the response was profoundly faster. Both changes were consistent with those seen in the single-pulse stimulation experiment (Fig. 3). Importantly, the extents of the changes in both peak current and activation time course were similar despite different time intervals between the conditioning pulse and the test pulse (Fig. 4 *B* versus Fig. 4 *D*). Thus the data suggest that the use dependence of the channel was long-lasting, with no significant recovery on a timescale of at least 30 min under these experimental conditions.

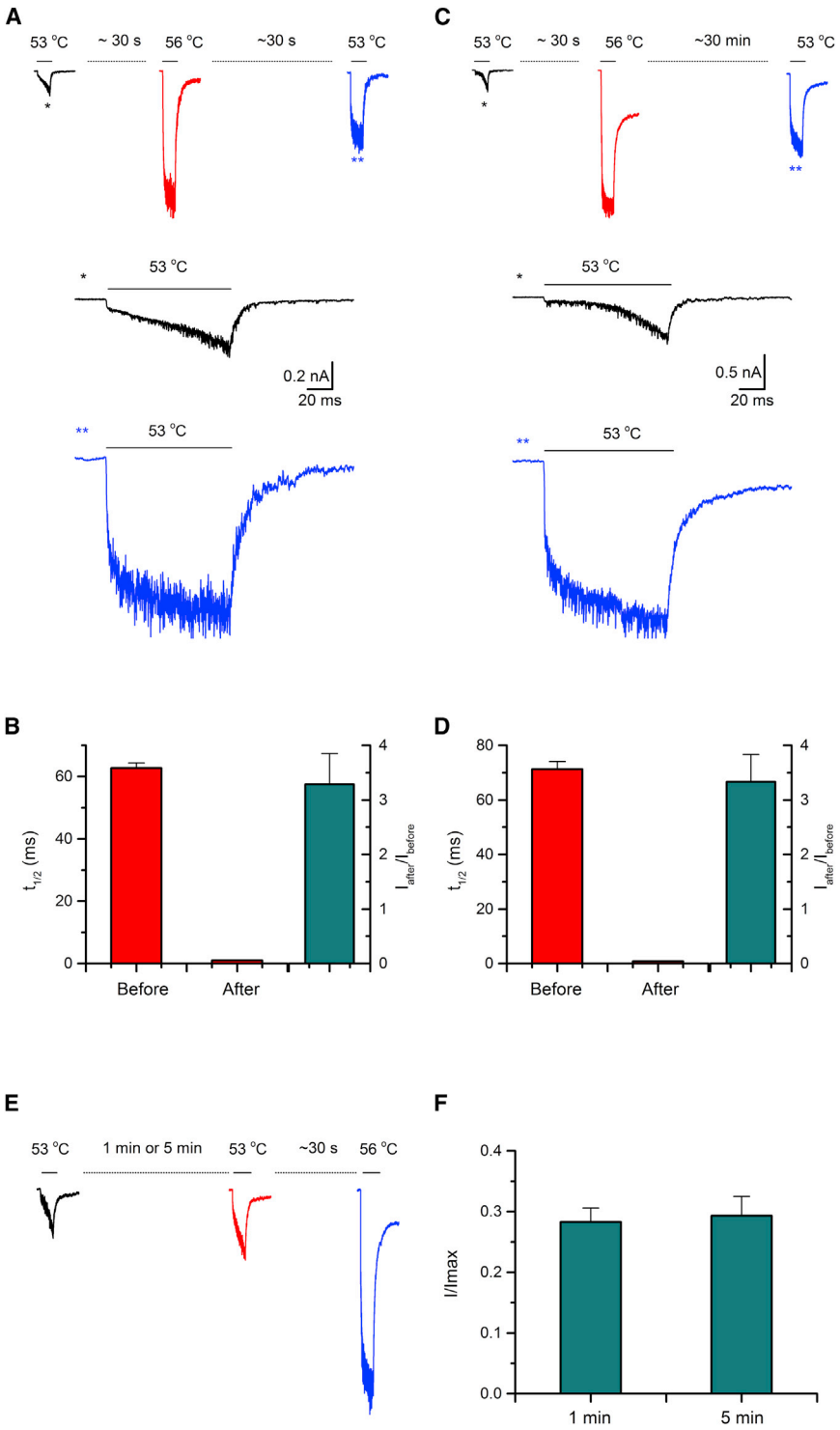

FIGURE 4 Irreversibility of use dependence. (*A*) Recordings showing responses to a test temperature pulse (53°C) before and after activation with a conditioning pulse (56°C). The time interval between two pulses was approximately half a minute. The inset traces below show an expanded view of the two test pulse responses at 53°C. (*B*) Statistical plots showing the half-activation time before and after application of the conditioning pulse (*left panel*) and the relative change of the peak current response (*right panel*). (*C* and *D*) Parallel plots for recordings where the time interval between the conditioning pulse and the second test pulse were increased to 30 min. (*E* and *F*) Recordings from inside-out membrane patches at −60 mV. To see this figure in color, go online.

Alteration of heat sensitivity underlies use dependence

In general, the slope of a temperature-responsiveness curve is correlated with the temperature dependence or the energetics of channel activation, i.e., the enthalpy change between closed and open conformations. According to this theory, the profound change in the slope sensitivity of TRPV2 during the initial and subsequent activations would be indicative of a loss of intrinsic temperature dependence or activation enthalpy of the channel. This would suggest a possible mechanism for the use dependence of TRPV2, where the temperature-sensing structures of the channel undergo changes so that it no longer confers a large enthalpy

for channel activation. However, the problem with this theory is that the gating during the initial activation of TRPV2 is not at equilibrium but involves irreversible changes (at least within the scale of experimental time). Thus, the apparent slope change may not be mechanistically corresponding to a change in the temperature-sensing energetics of the channel. Alternative analyses would be needed to quantify the intrinsic temperature dependence of TRPV2 as to whether it was indeed altered by stimulation.

We have estimated the temperature dependence of heat responses of TRPV2 by two means. In the first approach, we analyzed the activation time course explicitly with a kinetic model as we previously used for analysis of TRPV1/2 chimeric channels (18). The activation time course of TRPV2 has several features (Fig. 5 *A*). When temperature was just above the activation threshold, the current was activated slowly. When temperature was further elevated, the current involved a rapid rise at the beginning, followed by a slow rise. The current amplitude resulting from the rapid activation phase tended to be similar to the current response at the end of the previous temperature jump. A minimum model to capture these features would be a three-state model $C_1 \rightarrow C_2 \leftrightarrow O$, where the first transition from C_1 to C_2 accounts for irreversibility. The model assumes that the channel initially resided in C_1. The stimulation drove the channel away from C_1 into C_2, i.e., the C1 $\rightarrow$ C2 transition is temperature-dependent. The slow time course at low temperatures implies this transition to be slow compared to transitions of $C_2 \leftrightarrow O$. Thus, the initial activation from C_1 to C_2 is rate-limiting. As the channel became increasingly activated, the state C_2 became more significantly populated. Subsequent activations would involve two components—the activation from C_2, which was fast and accounted for the rapid rise of the current after a temperature jump; and the activation from C_1, which was slow and accounted for the slow time course of the response. Because the inverse transition from C_2 to C_1 is prohibited, the occupancy of C_2 depends on stimulation history and determines the amplitude of the fast activation phase after a temperature jump. The model thus has the essential features of the data.

Fig. 5 *A* shows the fit of initial responses over the first round of activation by the model. The model sufficed to capture the main features of the responses, including the initial fast rise of the current and the subsequent slow time course. The fitting corroborated that the transition from C_1 to C_2 was strongly driven by temperature, and underlined the sharp temperature dependence of the peak current of the channel. On average, the activation rate involved an enthalpy change 194 $\pm$ 8 kcal/mol ($n = 12$). The transitions between C_2 and O only needed to have nominal temperature dependence, which was negligible as compared to the C1 $\rightarrow$ C2 transition. Of note, the use of only two states (C_2 and O) to describe the gating of the channel after sensitization is an oversimplification. But the approximation has a limited impact on the estimation of the temperature dependence for initial activation because it is determined mainly by the slow activation from C_1 to C_2.

In the second approach, we found that the slope of the temperature-response curve of the peak current remained useful for approximating temperature dependence of the channel, although the early activation of TRPV2 was

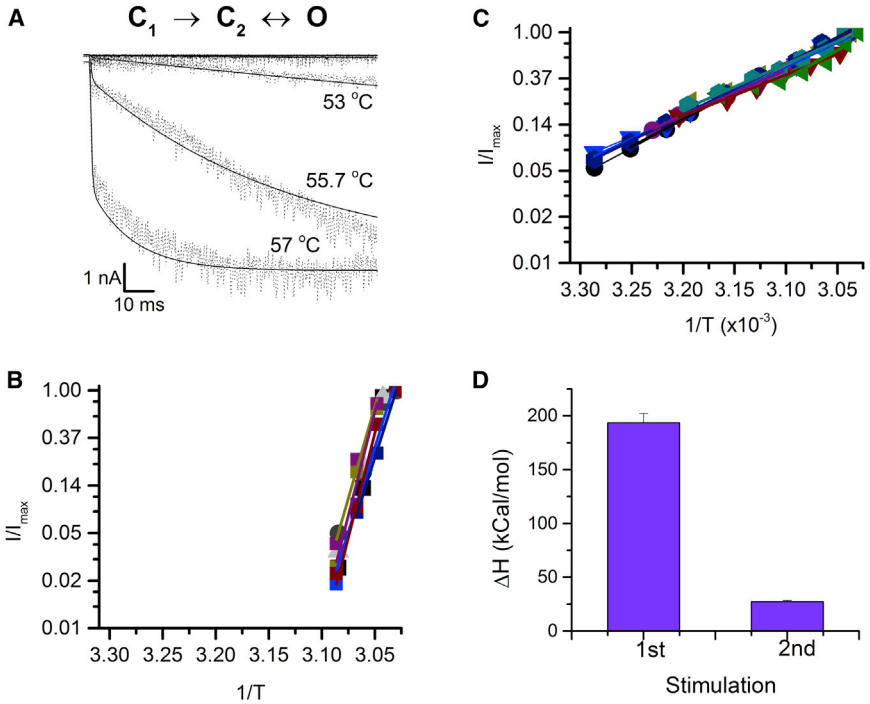

FIGURE 5 Alteration of intrinsic temperature dependence. (*A*) Model-based estimation of temperature dependence of TRPV2 during initial activation. The time course of activation was explicitly fit with a three-state model. The clean line in the plot shows the fit. (*B*) Estimation of temperature dependence from the current-temperature relationship. The peak current at the end of temperature pulse was plotted on the log scale versus 1/*T*. The rapidly rising phase during initial activation was fit linearly to estimate activation enthalpy. (*C*) Estimation of temperature dependence of TRPV2 during the second run of activation. The current-temperature relationship was fit by linear regression over the entire temperature range. (*D*) Comparison of temperature dependence over the first and second run of activation. The averaged enthalpy change between closed and open was plotted. To see this figure in color, go online.

deviated from the equilibrium. Fig. 5 *B* shows that the steepest portion of the curve attains an approximately linear relationship with respect to the reciprocal of temperature ($1/T$). A linear regression of the curve resulted in an enthalpy change $\Delta H = 186 \pm 14$ kcal/mol ($n = 12$). This estimate was lower than, but still in, the range of the model fit. The validity of the approach can be justified in theory with the aforementioned model as following. Because the transition $C_1 \rightarrow C_2$ was rate-limiting, the occupancies in C_2 and O were relatively equilibrated rapidly. As a result, the open probability could be simplified as $P_o = P_1\exp(-\alpha t)/(1 + K_d)$, where P_1 is the starting probability at state C_1 before a temperature jump, α is the activation rate from C_1 to C_2, and K_d is the open equilibrium constant between C_2 and O. During early activation (e.g., <56°C in Fig. 5 *A*), P_1 is close to unity. Under this condition and that K_d had nominal temperature dependence (relative to α), the temperature dependence of P_o would be dictated by the activation rate α from C_1 to C_2. This implies that the Arrhenius plot of the responses would still have a linear relationship and the slope is related to the temperature dependence of α. Thus the fitting of the slope of the peak response curve remained to provide an estimate for the temperature dependence of the channel.

After sensitization by stimulation, the channel was driven out of the initial state C_1 so its activation occurred between C_2 and O and therefore became reversible. To the first-order approximation, the responses obtained during the second run of stimulation could be considered to result from the postsensitization activation. Thus, the slope of the temperature-response curve during the second run provides a measurement for the temperature dependence of the channel after sensitization. Fig. 5 *C* shows the Arrhenius plot for the recordings over the second run of stimulation. The plot followed a linear trend with respect to the reciprocal of T over the entire temperature range (31–56°C). Linear fitting of the relationship resulted in an enthalpy change $\Delta H = 27 \pm 1$ kcal/mol ($n = 7$). This energetic value is similar to the energetic values of other ion channels with nominal temperature dependence, indicating that the channel had lost its large enthalpy change occurring during initial activation. These analyses show that the use dependence of TRPV2 is correlated to changes in the slope sensitivity of the temperature dependence of the channel. Heat stimulation can convert it from being extremely temperature-dependent to only nominally temperature-dependent.

Use dependence of agonist activation

Besides heat, vanilloid receptors are also activated by chemical agonists. We next examined whether the activation of TRPV2 by agonist was use-dependent. 2-APB is a common agonist of vanilloid receptors including TRPV2. Thus we tested the 2-APB response of TRPV2 over repeated stimulation. Fig. 6 *A* illustrates responses of the channel to 1 mM 2-APB. The current was recorded in an inside-out configuration to minimize other possible effects of 2-APB on intracellular proteins. When first applied, 2-APB evoked only a small current, but the response was increased progressively over repeated activation. Thus, the agonist response of TRPV2, like its heat response, was also use-dependent. On average, the increase of the current followed an approximately linear trend over 7–10 repetitions until finally reaching a steady state (Fig. 6 *B*). The final response was ~15 times the initial value, indicating that the channel could be significantly sensitized by stimulation.

In some experiments, we also observed that 1 mM 2-APB failed to illicit increased responses over repeated stimulation (Fig. 6 *C*). In these experiments, we further tested the sensitizability of the channel using a high dose for a maximum level activation. Fig. 6 *C* shows the protocol of stimulation and the corresponding current recordings for such experiments, where the response of the channel to 1 mM 2-APB was first measured, then 2-APB at saturating concentrations was applied to fully activate the channel, and finally the response to 1 mM 2-APB was assessed again. Here, even though the initial 1 mM 2-APB response was relatively large and stable, the subsequent stimulation at a high concentration could still led to a significant increase in the response (to 1 mM 2-APB). Thus, the channel remained sensitizable. On average, the increase of the response was greater than threefold ($n = 7$; Fig. 6 *D*). The reason for such variations in these experiments was unclear. But we noticed that in the latter case, the extent of the sensitization tended to be less than in the above experiments (Fig. 6 *D*) because the initial 1 mM 2-APB response was larger. It appeared that the channel in these experiments was upregulated so that the final extent of sensitization became less. Regardless of these quantitative differences, the use dependence of the activity was consistent.

Fig. 6, *E* and *F*, shows the measurement of the 2-APB dose-response curve of TRPV2 after sensitization. Here we first sensitized the channel by stimulation with saturating 2-APB, followed by measurement of 2-APB responses at different concentrations. The sensitized channel had an $EC_{50} = 0.74 \pm 0.08$ mM and a Hill coefficient $n_H = 2.43 \pm 0.33$ ($n = 6$). Also shown in Fig. 6 *F* is the control dose-response curve of 2-APB before sensitization (*black*), which could also be fit by a Hill equation with $EC_{50} = 1.21 \pm 0.02$ mM and $n_H = 3.43 \pm 0.18$ ($n \geq 6$), although the channel activation was out of equilibrium. The sensitization of the channel caused decreases in both EC_{50} and apparent cooperativity.

Cross sensitization of agonist response by heat stimulation

Because the channel exhibits use dependence in both agonist and heat responses, it raises the question whether the same mechanism underlies the use dependence. To address the issue, we investigated the cross dependences

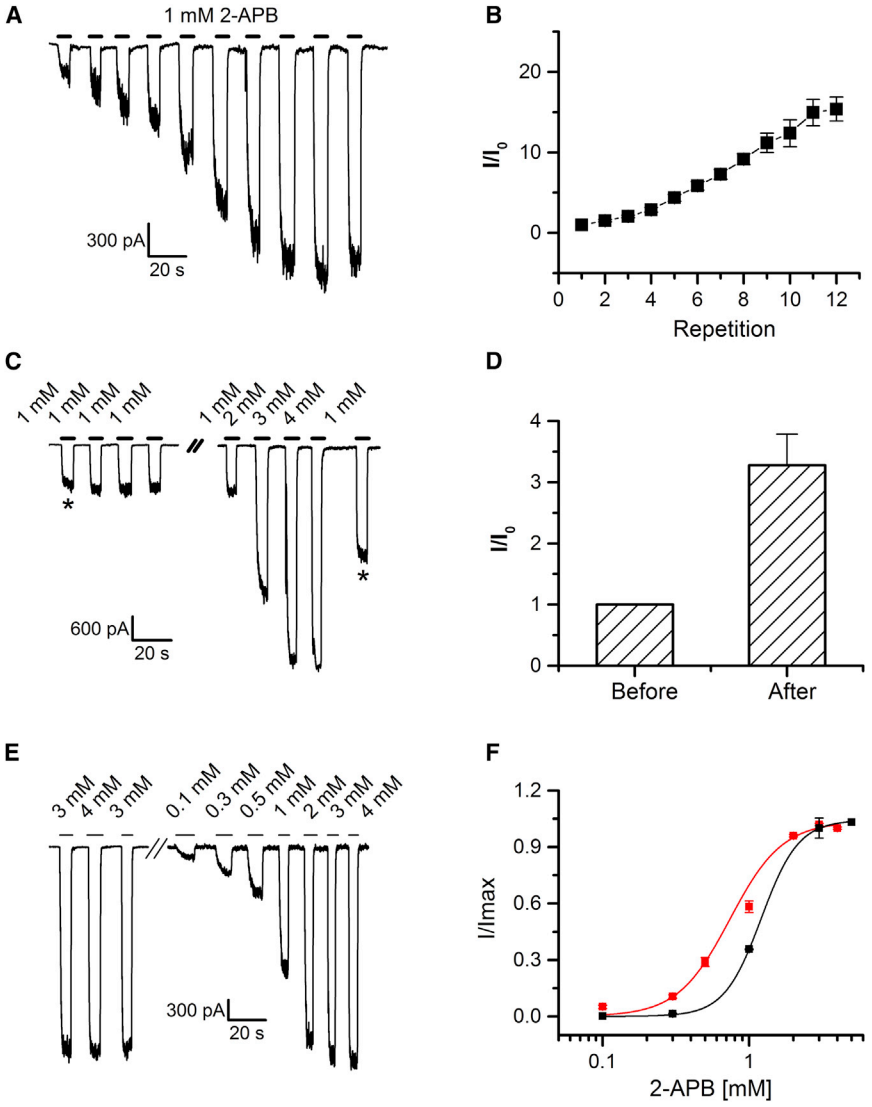

FIGURE 6 Use dependence of agonist activation. (*A*) Response of TRPV2 to 2-APB. The agonist was applied repetitively at the same concentration (1 mM). The response was increased progressively over repetitive stimulation before finally reaching a steady level. (*B*) Average plot showing current increase versus stimulation. The relative increase of the response with respect to the initial current was plotted. (*C*) Sensitization evoked by application of agonist at a high concentration. The recording shows relatively stable responses to 1 mM 2-APB initially, but after application of high concentrations of 2-APB, the response was further increased. (*D*) Statistical plot showing relative increase of 2-APB response (1 mM) before and after stimulation with saturating 2-APB. (*E* and *F*). Dose-response relationship. (*E*) Measurement of dose responsiveness. The channel was first sensitized by applications of saturating 2-APB at 3–4 mM, after which the responses to different concentrations of 2-APB were measured. (*F*) Dose-response curves of 2-APB before (*black*) and after (*red*) sensitization. The solid lines represent fits by Hill equations: $EC_{50} = 0.74 \pm 0.08$ mM, $n_H = 2.43 \pm 0.33$ ($n = 6$) (after) and $EC_{50} = 1.21 \pm 0.02$ mM, $n_H = 3.43 \pm 0.18$ ($n \geq 6$) (before). Recordings were from inside-out patches excised from HEK 293 cells transiently expressing rat TRPV2. Holding potential at −60 mV. To see this figure in color, go online.

between agonist and heat activations of TRPV2. We first examined the effect of heat stimulation on agonist activation. For these experiments we first measured the dose-response curve of 2-APB, then stimulated the channel with fast temperature jumps, and finally tested 2-APB dose responses again (Fig. 7 *A*). For heat stimulation, we exposed the patch to a family of temperature jumps ranging 43–53°C, each lasting 100 ms, similar to those used for heat activation in previous experiments. As evident from Fig. 7 *A*, the intervening heat stimulation had a profound impact on subsequent 2-APB responses. The channel began to respond to 2-APB at significantly lower concentrations (0.03 vs. 0.3 mM). Fig. 7 *B* plots the dose-response curve of 2-APB before and after heat stimulation (*cyan* versus *black*). Also plotted is the 2-APB dose-response curve after sensitization with 2-APB stimulation (*red*). The heat stimulation caused a profound shift of the dose-response curve, more than that induced by agonist stimulation. The fitting of the dose-response curves with Hill equations resulted in $EC_{50} = 0.25 \pm 0.03$ mM and $n_H = 1.53 \pm 0.23$ ($n = 6$) for heat treatment and $EC_{50} = 0.74 \pm 0.08$ mM and $n_H = 2.43 \pm 0.33$ ($n = 6$) for agonist treatment, respectively. Heat stimulation caused larger decreases in EC_{50} and in n_H than agonist stimulation. Thus, heat stimulation appeared more effective in sensitizing TRPV2 than agonist stimulation. The difference in the two suggests that their effects, albeit phenomenologically similar, may involve distinct mechanisms. Fig. 7 *C* compares the maximum response of 2-APB before and after heat stimulation (I_{after}/I_{before}). The postheat stimulation response became slightly reduced; however, the change was small and could be due to a loss of functional channels from excessive heating, and thus may not be mechanistically relevant. Overall, the channel retained a largely similar maximum response after heat stimulation, suggesting that the heat stimulation mainly impacts agonist responses in EC_{50} and n_H.

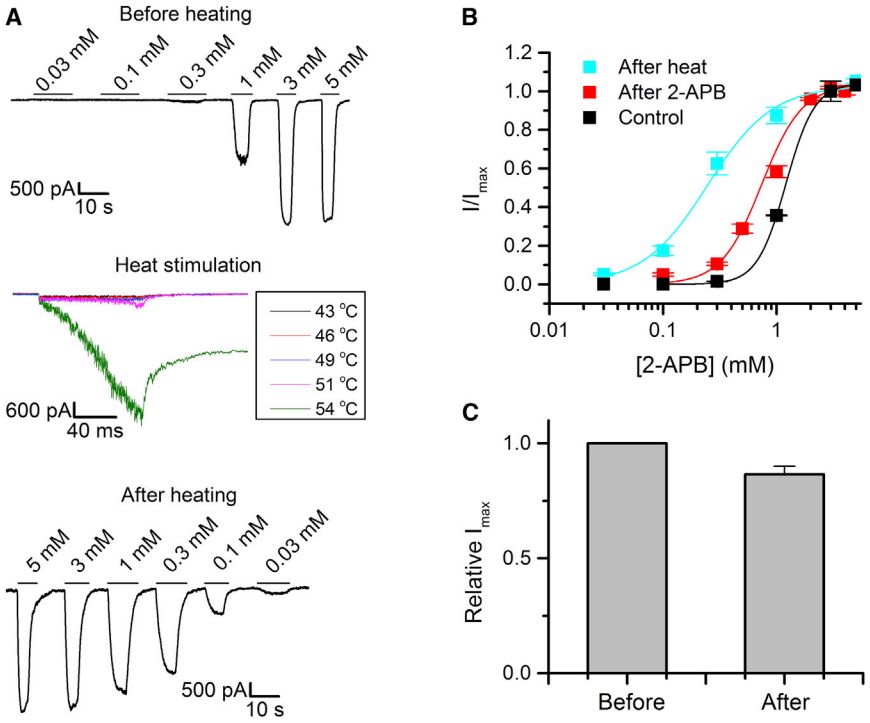

FIGURE 7 Cross sensitization of 2-APB responses by heat stimulation. (*A*) Current traces from the same patch showing stimulation protocols. (*Top*) The patch was first perfused with different concentrations of 2-ABP to measure the dose responses of 2-APB at control. (*Middle*) Fast temperature jumps were then applied to induce temperature-dependent sensitization. Temperature pulses ranged 43–54°C as indicated. (*Bottom*) 2-APB dose responses were measured again from the patch after heat stimulation. Recordings were from an inside-out patch of a HEK 293 cell expressing rat TRPV2. Holding potential at −60 mV. (*B*) Comparison of 2-APB dose-response curves before (*black*) and after (*cyan*) heat activation. Also shown is the 2-APB dose-response curve after sensitization by 2-APB as measured above (*red*). The Hill equation fit for the dose-response curve after heat sensitization corresponds to $EC_{50} = 0.35 \pm 0.03$ mM and $n_H = 1.53 \pm 0.23$ ($n = 6$), as compared to $EC_{50} = 0.74 \pm 0.08$ mM, $n_H = 2.43 \pm 0.33$ ($n = 6$) for 2-APB-induced sensitization. (*C*) Comparison of the maximum 2-APB response before and after heat stimulation. The relative change was plotted ($n = 5$). To see this figure in color, go online.

Agonist stimulation fails to impact heat sensitivity

Conversely, we examined whether prior agonist activation also influences the heat sensitivity of TRPV2. Fig. 8 *A* illustrates the experimental protocol where 2-APB was first applied to activate and sensitize the channel, followed by measurement of heat responses. The application of 2-APB was repeated until the response was maximally sensitized. While the final 2-APB response was significantly sensitized, the heat response of the channel attained similar characteristics to the control response without

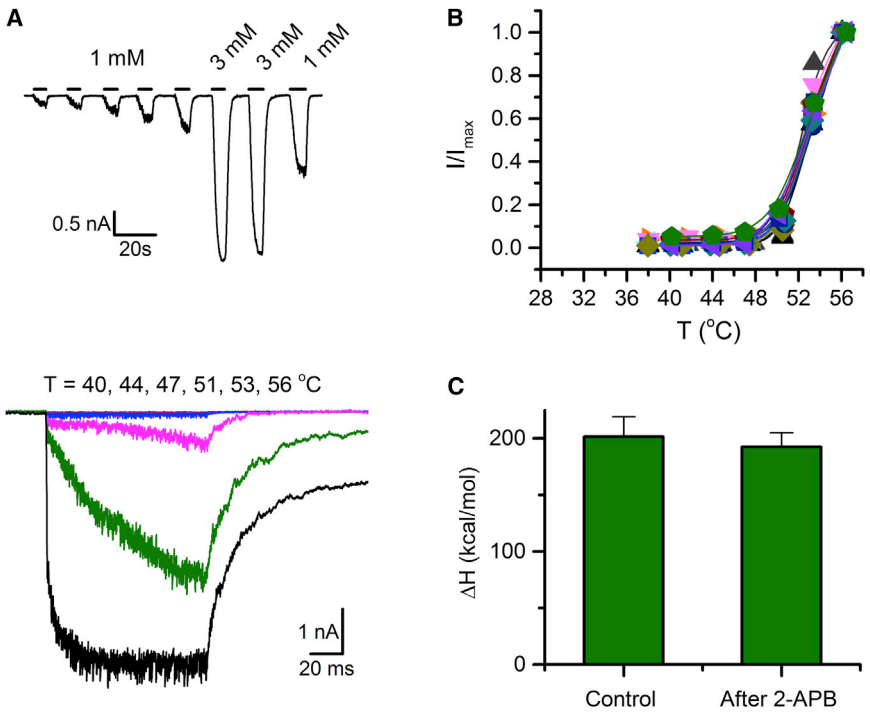

FIGURE 8 Heat activation after sensitization by agonist stimulation. (*A*) Current traces showing sensitization protocol. The channel was first sensitized by application of 2-APB (*top*), after which the heat response of the sensitized channel was measured using fast temperature jumps (*bottom*). The 1 mM 2-APB responses were compared before and after applications of saturating 2-APB to ensure that the channel was sensitized. (*B*) Temperature dependence of heat responses. (*C*) Comparison of the energetics of heat activation before and after sensitization by 2-APB. The energetics were estimated by linear fit of an Arrhenius plot of temperature responsiveness curves. Recordings were from transiently transfected HEK293 cells. Holding potential at −60 mV. To see this figure in color, go online.

2-APB treatment (Fig. 8 *A*). For example, the channel remained to have a high temperature activation threshold (~50°C) and a slow activation time course. Fig. 8 *B* plots temperature-response profiles after sensitization by 2-APB stimulation. Indeed, the current remained to be activated above 50°C, which is in contrast to warm temperatures when sensitized by heat (Fig. 1 *B*). The fitting of the slope of the curve resulted in $\Delta H = 193 \pm 13$ kcal/mol ($n = 10$), similar to the enthalpy change before sensitization (Fig. 8 *C*). Thus both the temperature responsive range and the temperature dependence of responses remained largely unchanged. The 2-APB stimulation, although it could sensitize its own response, had little effect on the heat sensitivity of TRPV2. This is opposed to the large impact of heat stimulation on 2-APB responses and suggests that different mechanisms may underlie the use dependence of heat and agonist responses of the channel.

Heat sensitivity of TRPV1 is stable

Lastly we asked whether the strong use-dependence of heat sensitivity as observed with TRPV2 is a general property of thermal channels. To address the issue, we examined another prototypical heat-sensitive channel, TRPV1, which shares a close homology with TRPV2. We first tested the stability of its heat sensitivity. Fig. 9 *A* illustrates heat-activated responses of TRPV1 evoked by two runs of repetitive stimulations, recorded in the same cell. In both cases, the activation began at ~41°C. Both the activation time course and current increment, with respect to temperature, were also similar between the two runs. One subtle difference is that there appeared to be larger responses at low temperatures (<41°C) during the second run. However, the currents remained small, so the changes could be due to leak currents, which usually became larger after high temperature exposures. Fig. 9 *B* plots the temperature-response profiles for control (*black*) and repeated (*red*) activations, respectively. The curves were nearly overlapping. On average, the enthalpy change as determined from the slope of the curves was $\Delta H = 97 \pm 8$ kcal/mol ($n = 5$) for the first run and $\Delta H = 84 \pm 9$ kcal/mol ($n = 5$) for the second run, respectively. The repeated activation involved a lower enthalpy change, but the change was subtle (Fig. 9 *C*), especially compared to changes of TRPV2. In addition to temperature dependence, the maximum heat response (at 51°C) was also only slightly reduced during the second run of stimulation (Fig. 9 *C*). The small change in the maximum response could be attributed to a loss of functional channels due to excess heat exposure. Overall, the heat activation of TRPV1 appeared to be relatively stable over repetitive stimulation, in contrast to that of TRPV2.

The 2-APB response of TRPV1, however, appeared differently. Fig. 9 *D* illustrates the 2-APB response of TRPV1 over repetitive stimulation at a subsaturating concentration. The response was increased progressively over repetitive stimulation, similar to the 2-APB response of TRPV2. The rate of the increase tended to be supralinear (Fig. 9 *E*), faster than the linear increase rate of TRPV2. The extent of the increase was also substantial, reaching ~15 ± 3 ($n = 6$) folds between the initial and the fully sensitized responses. These data indicate that the 2-APB response of TRPV1 is use-dependent. Thus the use dependence occurred differentially in TRPV1 to heat and 2-APB activations, which is consistent with the notion that the use dependence of heat and 2-APB activations is mechanistically different, as has been suggested for TRPV2.

We further examined whether the use dependence occurring to the 2-APB response is a general property of agonist activation of TRPV1. We tested another agonist of the channel, capsaicin, which has been shown to specifically bind to the channel. We first measured the capsaicin response of the channel at a subsaturating concentration (0.3 μM). Then the channel was treated with saturating capsaicin (3 μM), after which the low concentration response was tested again (Fig. 9 *F*). As observed with TRPV2, a full-level activation at saturating concentrations would incur a maximal sensitization if the response was use-dependent. However, as shown in Fig. 9, *F* and *G*, the subsaturating capsaicin responses remained largely unchanged before and after the intervening stimulation with saturating capsaicin. Thus, the capsaicin response differs from the 2-APB response; instead it resembles the heat response without significant use dependence.

DISCUSSION

A general conception on thermal channels is that each thermal channel responds to temperature over a specific range, which is correlated with a distinct thermal sensation of being noxiously cold, cool, warm, or noxiously hot. In contrast to this dogma, the activation of the vanilloid receptor TRPV2 exhibits strong use dependence. The use dependence of TRPV2 is manifested with several aspects of changes in its heat activation profiles. First, the temperature-responsive range of the channel or its activation threshold is greatly shifted from high noxious temperatures to warm temperatures. Second, the apparent temperature dependence or the slope of temperature responsiveness curve is significantly reduced. Third, the time course of activation becomes considerably faster. Our quantitative analyses, after taking account of the irreversibility of activation, indicate that such changes are correlated with specific activation steps pertinent to temperature sensing by the channel. In particular, the temperature dependence during the initial activation corresponds to a large enthalpy change in opening (ΔH ~ 193 kcal/mol). But after stimulation, the activation has only nominal temperature dependence. The stimulation causes a dramatic decrease in the activation enthalpy of the channel. The use dependence of TRPV2 thus unravels that temperature sensing by thermal

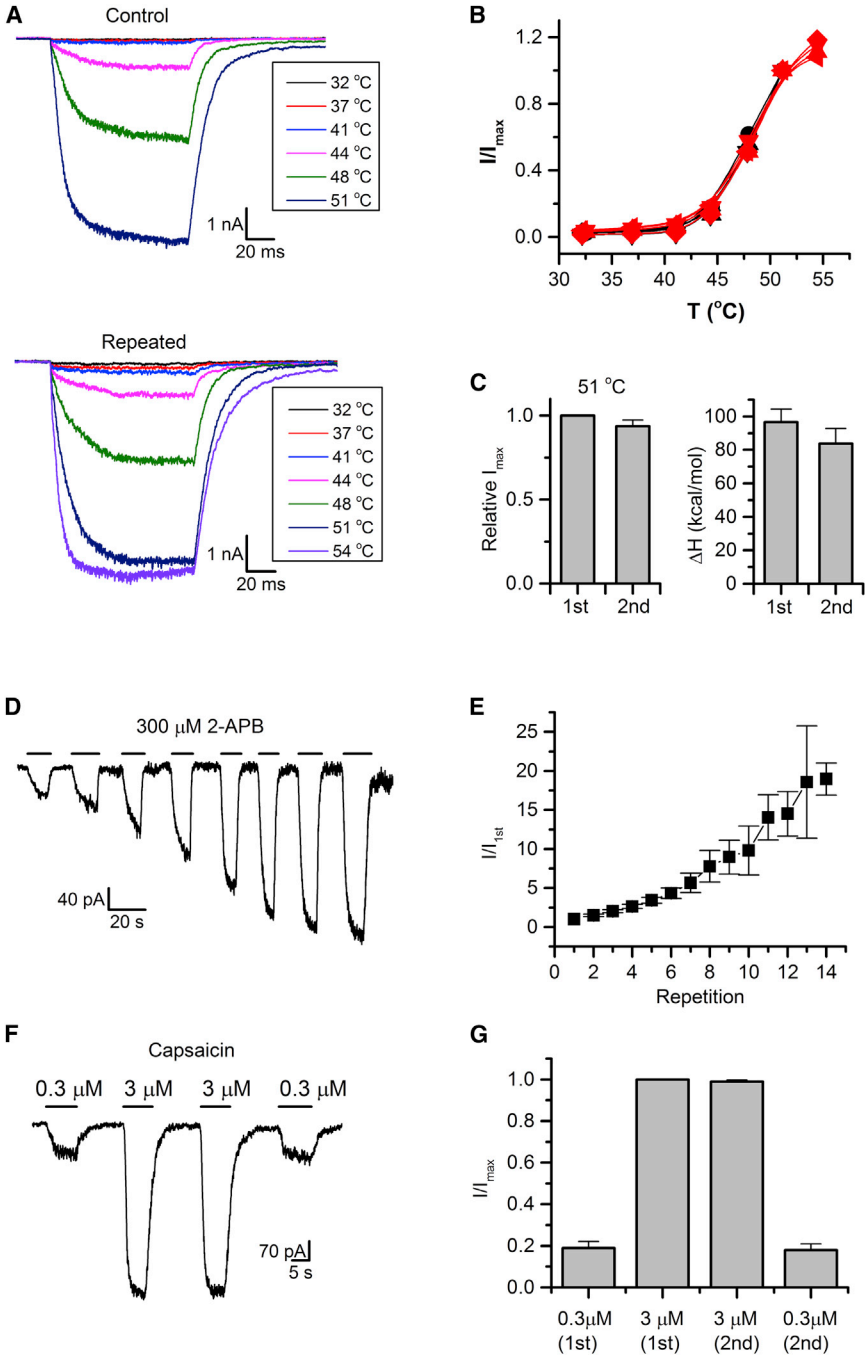

FIGURE 9 Stability of heat and agonist responses of TRPV1. (*A*) Temperature responses of TRPV1. Top traces correspond to the first run of heat activation, while bottom traces for the second run. The first run did not contain the maximum temperature jump to 54°C to improve the success rate of the second run. (*B*) Current-temperature relationship (*black*, control; *red*, repeated stimulation). (*C*) Comparison of the maximal heat response (*left*) and the activation energetics between the two runs. (*D*) Repetitive 2-APB responses of TRPV1, showing that the 2-APB activation was use-dependent. (*E*) Average plot showing relative increase of 2-APB response over repeated activation. Currents were normalized to initial 2-APB responses. (*F*) Recordings of capsaicin responses, showing subsaturating capsaicin (0.3 μM) currents before and after activation by saturating capsaicin (3 μM). (*G*) Comparison of capsaicin responses between stimulations. Data were averaged from 10 experiments. Recordings were from inside-out patches at −60 mV. To see this figure in color, go online.

channels can be highly dynamic. Remarkably, despite a nearly complete loss of the strong temperature dependence (i.e., the large enthalpy change between closed and open), the channel remains activated by heat, which argues that the pore opening stays intact. This would be consistent with the notion that temperature sensing and pore opening are two separate processes.

The agonist-dependent activation of TRPV2 also displays use dependence. It leads to a significant leftward shift in the dose-response curve, which effectively sensitizes channel responses. The sensitization can be induced by repeated stimulation at a low concentration or a single application at a high concentration. It appears that the sensitization occurs irrespective of the stimulation protocol; instead it depends on the level of opening. The use dependence of agonist response is, however, distinct from that of heat response. This is evident from the cross-use dependence between the two stimuli. Prior activation by heat could alter subsequent responses of the channel to both heat and agonist. However, the activation by agonist could only sensitize subsequent agonist responses but exerted little effect on heat responses. In particular, the large enthalpy

change associated with heat activation stays unchanged even though the channel has been sensitized for agonist activation. Because a large enthalpy change is the determinant of high temperature dependence, this suggests that the mechanism for temperature sensing by the channel remains intact despite alteration of agonist sensitivity. In other words, the process of temperature sensing in the channel involves a different pathway from agonist activation, which is consistent with the notion that the heat sensitivity of the channel is confined within the channel.

The studies on heat sensitivity of TRPV2 are relatively few in the literature and mostly focus on the characterization of whether TRPV2 underlies the high-threshold heat response of nociceptors. From these studies it appears to be inconclusive whether heat stimulation can sensitize TRPV2 (3,4,9,11,29–31) and if so, what mechanisms are involved (9–11). For example, Ahluwalia et al. (9) and Rau et al. (11) show that the heat-induced sensitization is dependent on intracellular calcium, whereas Leffler et al. (10) suggests the opposite. Our results concur that the heat response of TRPV2 can be strongly sensitized by repetitive stimulation. Mechanistically, our experiments were conducted in a nominally calcium-free condition and thus would support a calcium-independent mechanism. To this end, we find that the use dependence of TRPV2 is reminiscent of the stimulation-dependent sensitization of another heat-sensitive channel TRPV3. One explanation for the sensitization of TRPV3 was a voltage-dependent pore block by divalent cations such as calcium (32). The sensitization of TRPV3 was also found to be differentially influenced by calcium chelators EGTA and BAPTA, which led to the proposition of Ca^{2+}-dependent intracellular regulation such as CaM modulation of the channel (33). A more recent study, however, shows that the sensitization of TRPV3 occurs due to hysteresis of gating, and thus is intrinsic to the channel itself (34). The use dependence of TRPV2 also persisted in excised membrane patches. These similarities suggest that the use dependence of TRPV2 may also occur from changes at the level of gating of the channel.

Given the occurrence of strong use dependence in both TRPV2 and TRPV3, it is tempting to speculate whether the use dependence is a common property of thermal channels. This promoted us to examine another homolog, TRPV1. In contrast, the heat activation of TRPV1 was stable, showing no significant changes in temperature activation profiles over repetitive stimulation. Whether TRPV1 or TRPV1-containing low-threshold nociceptors are susceptible to sensitization by repetitive heat stimulation, has been controversial (3,4,6,29,30,35,36). The reason for the discrepancy is unknown but may be related to stimulation protocols because slow excessive heating may adversely alter biophysical properties of membranes or proteins. Our result indicates that when stimulated by short temperature pulses, the heat sensitivity of the channel is stable. The agonist responses of TRPV1 also differ in use dependence. Like the heat response, the capsaicin response of the channel exhibited no significant use dependence. However, the response evoked by 2-APB still possesses a considerable degree of use dependence, like those of TRPV2 and TRPV3. These observations argue that the heat-sensitive use dependence does not pertain to all thermal channels; instead, it appears to be specific to TRPV2 and TRPV3. The differential occurrence of the use dependence to the 2-APB response of TRPV1 also supports that the use dependence with different stimuli involves different mechanisms, consistent with the finding in TRPV2 that the heat activation sensitized the 2-APB response but not conversely.

The precise mechanisms and the structural basis of such use dependence as occurring in TRPV2, however, remain to be determined. One possible explanation is that the channel is structurally flexible. Our data suggest that the use dependence of TRPV2 could be modeled by a three-state model. According to this model, the channel has two resting states with different thermal stability. Freshly synthesized channels in cells are at a thermally less stable state so that heat stimulation can covert the channel to a thermally more stable structure. This level of structural flexibility may be related to the strong temperature dependence of these channels. According to the thermodynamic theory, the temperature dependence of a channel is determined by enthalpy and entropy changes between closed and open conformations. Thus, the strong temperature dependence of thermal channels requires that the channels are capable for large enthalpy and entropy changes, and the latter are usually correlated with a high degree of structural flexibility. On the other hand, an exceedingly large energetics value may incur excessive structural changes, which may compromise reversibility of conformational changes. In this regard, we noticed that TRPV2 involves an enthalpy change approximately twice that of TRPV1, while the enthalpy change of TRPV1 is already considered to be exceedingly large, approximately five times that of ligand- or voltage-gated channels. Thus, it is conceivable that the opening of TRPV2 involves such substantial structure changes that they cannot be entirely reversed upon channel closing. This would also explain why the heat activation of TRPV1 is not use-dependent, possibly because its energetics value is still within the limit for reversible transitions. The energetics values of TRPV3 before and after sensitization have not been well established. It would be interesting to see whether it is as large as that of TRPV2, which would provide further insights on whether the use dependence of these channels is correlated to their unusually strong temperature dependence.

The use dependence of the agonist activation of TRPV2 may be also related to the structural flexibility of the channel. As noted above, the difference in the cross use dependence with different stimuli (agonist versus heat) implies that the heat and agonist activations involve different pathways within the channel. The structural changes associated

with the use dependence of heat activation are presumably more extensive than those for agonist activation. This could mean that heat stimulation affects subsequent agonist responses as well. On the other hand, the structural changes associated with the use dependence of agonist activation may be more localized to the agonist activation pathway; or even if it crosses over to heat activation pathway, it does not incur large enthalpy and entropy changes sufficient to significantly perturb the strong temperature dependence of heat activation. As a result, the agonist activation would have a minimal impact on subsequent heat activation. By an allosteric model, the gating by an agonist involves agonist binding, intrinsic pore opening and closing, and allosteric coupling between them. Because the pore opening is also involved in heat activation, its change would have altered both heat and agonist sensitivity. Thus the use dependence of the 2-APB response is probably not due to alteration of intrinsic pore opening; instead it is more likely related to changes in allosteric coupling or agonist binding. However, we must be also cautious about the assumption that 2-APB has discrete binding sites on the channel implicit to such models. The concentration of 2-APB for TRPV2 activation is relatively high in the millimolar range. The compound is highly hydrophobic. Thus, it may nonspecifically interact with the channel in multiple manners. For example, it may be associated to the channel at hydrophobic pockets or binds to transmembrane domains of the channel to exert its effects. Alternatively, it may also partition into membranes to alter the state of lipid bilayer. If the association/disassociation rates of the compound to/from these sites are slow, a use-dependent activity would be manifest. Such nonspecific effects could also explain why they do not impact heat activation, presumably because they have a limited energetics contribution to the large energetics basis of temperature activation of the channel. They would be also consistent with the findings with TRPV1, where the use dependence occurs for the 2-APB response but not the response to capsaicin, an agonist with a specific binding site on the channel.

The use dependence as manifested in TRPV2 brings a unique mechanism for sensitization of thermal channels, which can further broaden channel functions. The sensitization mechanisms of thermal channels are perhaps best studied for TRPV1, which has been found to be the downstream target for regulation by many secondary messengers, especially inflammatory mediators released after tissue injury and inflammation such as prostaglandins, bradykinin, ATP, protons, nerve growth factors, and so on (37). The upregulation of TRPV1 by these inflammatory molecules serves a model mechanism for development of enhanced pain sensitivity, especially thermal hyperalgesia (38). In contrast to TRPV1, the regulation of TRPV2 is less well understood. However, single fiber recordings have shown that peripheral thermal hyperalgesia involves sensitization of both CMH and AMH I nociceptors (1) in various species. While TRPV1 sensitization by inflammatory mediators can account for the contribution of the CMH nociceptor, the hyperalgesia mediated by the AMH I nociceptor likely involves the vanilloid receptor TRPV2. The sensitization of the AMH I nociceptor has been characterized mainly by a large reduction in temperature threshold after heat stimulation (1), which is parallel to the use-dependent change in heat response of TRPV2. Thus, the use dependence of TRPV2 could account for the sensitization of the AMH I nociceptor. Such use-dependent sensitization does not require release of secondary mediators, suggesting that fundamentally distinct mechanisms may underlie TRPV1- and TRPV2-mediated thermal hyperalgesia.

AUTHOR CONTRIBUTIONS

B.L. designed research, performed research, and analyzed data; and F.Q. designed research, analyzed data, and wrote the article.

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