Heat activation is intrinsic to the pore domain of TRPV1

Feng Zhang\textsuperscript{a}, Andres Jara-Oseguera\textsuperscript{a}, Tsg-Hui Chang\textsuperscript{a}, Chanhyung Bae\textsuperscript{b}, Sonya M. Hanson\textsuperscript{a,1}, and Kenton J. Swartz\textsuperscript{a,2}

\textsuperscript{a}Molecular Physiology and Biophysics Section, Porter Neuroscience Research Center, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

Edited by Ardem Patapoutian, Scripps Research Institute, La Jolla, CA, and approved November 30, 2017 (received for review September 29, 2017)

The TRPV1 channel is a sensitive detector of pain-producing stimuli, including noxious heat, acid, inflammatory mediators, and vanilloid compounds. Although binding sites for some activators have been identified, the location of the temperature sensor remains elusive. Using available structures of TRPV1 and voltage-activated potassium channels, we engineered chimeras wherein transmembrane regions of TRPV1 were transplanted into the Shaker Kv channel. Here we show that transplanting the pore domain of TRPV1 into Shaker gives rise to functional channels that can be activated by a TRPV1-selective tarantula toxin that binds to the outer pore of the channel. This pore-domain chimera is permeable to Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} ions, and remarkably, is also robustly activated by noxious heat. Our results demonstrate that the pore of TRPV1 is a transportable domain that contains the structural elements sufficient for activation by noxious heat.

capsaicin | transient receptor potential | thermoensing | tarantula toxin | DkTx

Temperature-sensitive transient receptor potential (TRP) channels play critical biological roles in sensing heat and cold (1, 2). The vanilloid-sensitive TRPV1 channel can be activated by noxious or pain-producing stimuli, including heat, vanilloids such as capsaicin from hot chili peppers, extracellular acid, and venom toxins (3). Vanilloids bind to the intracellular ends of the S3–S6 transmembrane (TM) helices, whereas the TRPV1-specific double-knot toxin (DkTx) from tarantula venom and protons bind to the extracellular end of the pore formed by the S5–S6 helices (4–6). In contrast, localizing the temperature-sensing elements within TRPV1, as well as those within all other temperature-activated TRP channels, has not yet been achieved (1). Recordings of purified TRPV1 channels reconstituted into artificial liposomes suggest that the protein is intrinsically heat sensitive (7), and manipulating the lipid and cholesterol composition of native membrane suggests that lipids likely do not play a critical role in temperature sensing (8). In contrast, mutagenesis or deletion studies have identified several regions that are important for heat-dependent activation throughout TRPV1 and other heat-activated TRP channels. These regions include the N-terminal ankyrin repeat domains in TRPA1 (9), a membrane-proximal N-terminal segment for TRPV1, TRPV2, and TRPV3 (10, 11), the C terminus of TRPV1 (12–15), and the pore domain in TRPV1 (16–19), TRPV3 (20), and TRPA1 (21, 22) channels. Indeed, it has been proposed that residues involved in temperature sensing may be scattered throughout the receptor rather than forming a defined temperature-sensing domain (23). Although these studies identify regions of the protein where manipulations alter heat activation, it remains unclear whether they contribute directly to temperature sensing or have other roles in allosteric coupling or opening and closing of the pore. Furthermore, truncation of most of the N and C termini of TRPV1 cannot be achieved without losing channel function (13, 24), so it has not been possible to evaluate whether the cytoplasmic domains are essential for the temperature-sensing mechanism. We were particularly interested in exploring the role of the pore domain of TRPV1 in temperature sensing because heat activation of the channel is exquisitely sensitive to binding of Na\textsuperscript{+}, H\textsuperscript{+}, or DkTx to the external pore (19, 25). In the present study, we take advantage of the overall structural similarities of the pore domains of TRPV1 and voltage-activated K\textsuperscript{+} channels (4, 26) to generate chimeras in which the pore domain of TRPV1 was transferred into the Shaker K\textsuperscript{+} channel. We succeeded in generating functional chimeras, one of which can be activated by TRPV1-selective tarantula toxins, displays Ca\textsuperscript{2+} permeability and remarkably can also be activated by noxious heat with similar sensitivity to that of the TRPV1 channel.

Results

If the pore domain of TRPV1 contains the temperature sensor, we reasoned that it may be possible to generate heat-activated chimeras by transplanting the pore domain of TRPV1 into a structurally related channel whose activity lacks strong temperature sensitivity. To test this idea, we selected the Shaker K\textsuperscript{+} channel, a well-studied voltage-activated K\textsuperscript{+} (Kv) channel that is relatively temperature insensitive (27), making it an excellent background in which to evaluate temperature-sensitive elements in TRPV1. Alignment of the crystal structure of a channel construct closely related to Shaker, the Kv1.2/2.1 paddle chimera (26), to a cryo-EM structure of TRPV1 (4) reveals that the backbone of their integral membrane domains (S1–S6) is remarkably similar, with especially low C\textsubscript{rmsd} (2.5 Å) and high TM score (0.75) within the pore-forming regions (Fig. 1L and Materials and Methods) (28). Using backbone superposition of the TRPV1 and Kv1.2/2.1 structures to align the primary sequences of TRPV1 and the Shaker Kv channel (Fig. 1L and Materials and Methods), we designed chimeras in which varying portions of the S1–S6 helices were transplanted between Shaker and TRPV1 (Fig. S1). We then expressed each of the chimeras in Xenopus oocytes and tested for activation of membrane currents in response to voltage steps or to the extracellular application of a more potent variant of DkTx that contains two high-affinity K2 knot (K2K2) (5). Although most chimeras failed to give rise to functional channels, we identified one functional chimera in

Significance

The TRPV1 channel is an important detector of noxious heat, yet the location of the heat sensor and the mechanism of heat activation remain poorly understood. Here we used structure-based engineering between the heat-activated TRPV1 channel and the Shaker Kv channel to demonstrate that transplantation of the pore domain of TRPV1 into Shaker gives rise to functional channels that can be activated by a TRPV1-selective tarantula toxin and by noxious heat, demonstrating that the pore of TRPV1 contains the structural elements sufficient for activation by temperature.

Author contributions: F.Z., A.J.-O., S.M.H., and K.J.S. designed research; F.Z., A.J.-O., T.-H.C., and K.J.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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1Present address: Computational Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065.

2To whom correspondence should be addressed. Email: Kenton.Swartz@nih.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1717192115/-/DCSupplemental.
which the entire S5–S6 region was transferred from TRPV1 into Shaker (C11) (Fig. 1B) and another including an additional short extension past the C terminus of the TRPV1 S6 helix (C9) (Fig. S1). We chose to focus on the smaller C11 chimera, which exhibited robust activation of membrane currents in response to K2K2 application, with relatively linear current–voltage (I–V) relations (Fig. 1C–E). Both Shaker and the C11 chimera traffic to the plasma membrane, as surface biotinylation of intact cells with a membrane-impermeant reagent led to specific streptavidin-mediated pulldown of the proteins following detergent solubilization (Fig. S2). The C11 chimera could also be activated by DTX (Fig. S3 A and B), but not by the vanilloid resiniferatoxin (RTX) (Fig. 1C), as expected because this chimera does not contain the most critical residues for vanilloid sensitivity (29). The C11 chimera was inhibited by ruthenium red (RR), a nonspecific inhibitor of TRPV1 channels (30), although the extent of inhibition was less for C11 compared with TRPV1 (Fig. S3C). In addition, extracellular Zn²⁺ inhibited TRPV1 and the C11 chimera with similar affinity but had no apparent effect on Shaker (Fig. 1C and F and Fig. S4 A–D), suggesting that the metal binds within the pore domain of TRPV1. The C11 chimera was not blocked by Agitoxin-2 (AgTx-2; Fig. 1C and F), a selective pore-blocker of Shaker (31, 32), confirming that the functional pore of the chimera is derived from TRPV1 rather than Shaker. We also expressed the C11 chimera in HEK cells, and observed that cells looked unhealthy within 24 h and that it was not possible to obtain the GΩ seals required for stable whole-cell recordings. Because oocytes better tolerate the expression of Ca²⁺ permeable channels on their plasma membrane, and expression levels can be readily controlled by titrating the concentration of cRNA injected, we used oocytes for further characterization of the C11 chimera.

Although our results thus far demonstrate that the C11 chimera forms functional ion channels, we were concerned that the structure of the permeation pathway might be distorted compared with TRPV1. The TRPV1 channel is considerably more permeable to small monovalent cations such as Na⁺ compared with the larger organic cation N-methyl-d-glucamine (NMDG⁺) and is even more permeable to Ca²⁺ (30, 33). To investigate the ion selectivity of the C11 chimera, we first obtained I–V relations in the presence of either external K⁺, Na⁺, or NMDG⁺ (all in the absence of Ca²⁺) after activating the channel with K2K2. For both TRPV1 and the C11 chimera, I–V relations reversed at $V_{\text{rev}}$, values close to 0 mV in external solutions containing 100 mM K⁺ or Na⁺ (Fig. 2 A and B and Fig. S4 E and F), as expected because the internal K⁺ concentration in these cells was about 100 mM and the TRPV1 channel has similar permeability to Na⁺ or K⁺ ions (30). We then replaced external K⁺ with NMDG⁺ and observed large shifts of the I–V relations to negative voltages for TRPV1 and the C11 chimera (Fig. 2 A and B), indicating that both channels are considerably more permeable to K⁺ compared with NMDG⁺. To test whether the C11 chimera was permeable to Ca²⁺, we examined whether its activation would allow Ca²⁺ to enter oocytes and open Ca²⁺-activated

![Fig. 1. Transfer of the pore domain from TRPV1 into the Shaker Kv channel. (A) Backbone superposition of the pore domains of the Kv1.2/1.1 paddle chimera (gray, PDB ID code 2R9R) and RTx/DKtx-bound TRPV1 (cyan; PDB ID code 5R8X) using TM align (28). Backbone rmsd = 2.5 Å and TM score = 0.75. (B) Structure-based sequence alignment of Shaker (black), TRPV1 (red), and the C11 pore chimera in the transmembrane region spanning from S4 through S6 with conserved residues shown in bold. (C) Representative time courses for recording membrane currents at −60 and +60 mV from an un.injected oocyte (Left: control) and an oocyte injected with the C11 chimera (Right). Cells were held at −60 mV and voltage was stepped to −60 mV for 100 ms at 3-s intervals. Mean inward current at −60 mV (Lower symbols) and outward current at +60 mV (Upper symbols) are plotted vs. time.

RTX (100 nM), K2K2 (5 μM), AgTx-2 (100 nM), and Zn²⁺ (2 mM) were applied to the extracellular solution as indicated by horizontal bars. Dotted blue lines represent the zero-current level. The external recording solution contained (in millimoles/liter): 100 KCl, 10 Hepes, 2 MgCl₂, pH 7.4. (D) Representative current traces from control (Top) and C11 chimera (Bottom) before (Left) and after (Right) the addition of 5 μM K2K2 ($V_{\text{rev}} = −60$ mV, test depolarizations from −60 to +60 mV in 10-mV increments). (E) I–V relations for membrane currents recording in the presence of 5 μM K2K2 in control uninjectted oocytes (black symbols) and C11 injected oocytes (red symbols) obtained from experiments as in D (mean ± SEM for n = 4). (F) Normalized I–V relations before (black symbols) and after K2K2 application (5 μM, red symbols), followed by application of AgTx-2 (100 nM, green symbols) and then Zn²⁺ (2 mM, blue symbols), obtained from experiments as in D. Currents were normalized to the amplitude of current in the presence of 5 μM K2K2 at +60 mV (mean ± SEM for n = 7).
Cl\(^{-}\) channels that are native to these cells (34). When cells expressing TRPV1 or the C11 chimera were placed in solutions containing external Ca\(^{2+}\) ions, the Ca\(^{2+}\)-activated Cl\(^{-}\) channel inhibitor CaccinH-A01 (abbreviated A01) (35) inhibited a fraction of the K2K2-activated membrane current (Fig. 2 C and E). In contrast, A01 had no detectable effect when the external solution did not contain Ca\(^{2+}\) ions, or when cells were injected with the Ca\(^{2+}\) chelator BAPTA (Fig. 2 D and E). From these results, we conclude that the C11 chimera is Ca\(^{2+}\) impermeable.

The C11 chimera retains the S1–S4 voltage-sensing domains from Shaker, yet macroscopic currents measured for C11 exhibited little voltage dependence (Fig. 1 D and E), suggesting that the voltage sensors are not tightly coupled to the pore domain in this chimera. Tight packing between the internal end of S6 and the S4–S5 linker has been shown to be critical for such coupling in Kv channels (36, 37), and these contacts would likely be disrupted in the C11 chimera because it contains the S6 helix from TRPV1 and an S4–S5 linker helix from Shaker. To explore whether the S1–S4 domain has any functional role in the C11 chimera, we investigated the impact of modifying the S3b–S4 voltage-sensor paddle motif (38–41). Neutralization of the outer three S4 Arg residues in Shaker leads to constitutive activation of the channel (42, 43), suggesting that these S4 charges are required for negative membrane voltages to drive the voltage sensors into resting states and to close the pore. The S3b–S4 paddle motif in Shaker contains the three outer S4 Arg residues, and when we replaced the paddle of Shaker with the corresponding region of TRPV1 to generate chimera 21, we observed constitutive activation of the channel that could be fully inhibited by AgTx-2 (Fig. 3 A–C). We then made the same replacement of the S3b–S4 paddle of Shaker in the background of C11 to generate the C23 chimera and observed K2K2-activated currents that were indistinguishable from those of the C11 chimera (Fig. 3 A and D–F). This result is important because it suggests that S1–S4 voltage-sensing domains of Shaker do not have detectable influence on the pore domain of TRPV1, as if Shaker simply serves as a scaffold for expressing functional pore domains of TRPV1 to the plasma membrane. We note that both the C11 and C23 chimeras exhibited small voltage-dependent relaxations at the most positive membrane voltages studied (see Fig. 1 D for C11 and Fig. 3 E for C23), suggesting that the weak voltage sensitivity that is a hallmark of TRPV1 (44) may be partly maintained in the C11 chimera.

The results thus far show that transfer of the pore domain of TRPV1 into Shaker gives rise to small-cation selective channels that can be activated by K2K2. To explore whether the pore domain of TRPV1 contains the necessary elements for sensing noxious heat, we undertook temperature-activation experiments on control cells not expressing exogenous channels and compared them with those expressing either TRPV1 or the C11 chimera. We observed robust activation of membrane currents by heat for cells expressing TRPV1 (Fig. 4 A–C), consistent with earlier studies (30). In contrast, we observed little if any activation of membrane currents in uninjected cells (Fig. 4 D–F), or cells expressing either Shaker (Fig. S5 A–C) or Shaker ILT (Fig. S5 D and E), a Shaker mutant reported to facilitate observation of intrinsic temperature-dependent gating (45). Remarkably, expression of the C11 chimera gave rise to robust activation of membrane currents by noxious heat (Fig. 4 G–I), and these could be reversed by subsequent cooling or inhibited by extracellular application of Zn\(^{2+}\) while maintaining the heat stimulus (Fig. 4 J and K). In addition, the C23 chimera lacking the three outer S4 Arg residues retained robust activation by noxious heat (Fig. 4 L–N), confirming that the voltage-sensing domains in Shaker do not play an important role in the sensitivity of these chimeras to heat. To compare the steepness of temperature-activation relations for TRPV1 and the chimeras in a semiquantitative manner, we determined Q\(_{10}\) values for activation of membrane currents above 36 °C and obtained values of 26 ± 7.4 for TRPV1, 40.8 ± 7.5 for the C11 chimera, and 29.8 ± 9.7 for the C23 chimera (Fig. 4 C, I, and N), values that are in agreement with reported heat
sensitivities of TRPV1 in this temperature range (8, 19, 46). In contrast, the steepness of the relations for cells expressing TRPV1, C11, or C23 at lower temperatures (~15–30 °C; where leak currents make a major contribution to the recorded currents), was very small and similar to the Q10 values at T > 37 °C for uninjected oocytes and oocytes expressing Shaker and Shaker ILT (Fig. 4F and Fig. S5 C and F). Although these data do not rule out the additional involvement of other regions, they suggest that the pore domain of TRPV1 contains the structural elements that are required for activation by noxious heat with a similar sensitivity as the full-length TRPV1 channel.

To test whether pore domain mutants established to perturb gating in TRPV1 would cause similar perturbations in C11, we studied the F640L mutant that stabilizes the open state, causing partial constitutive activation of TRPV1, enhanced sensitivity to capsaicin, and heat-sensitive currents at lower temperatures than those required to activate the WT channel (16). For both TRPV1 and the C11 chimera, we observed that the mutation caused partial constitutive activation of the channel at room temperature (Fig. 5 A and B), could be further activated by capsaicin or K2K2 (Fig. 5 A and B), and displayed clearly altered responses to heating (Fig. 5 C and D). From these results, we conclude that the F640L mutant similarly stabilizes the open state of the pore domain in both TRPV1 and the C11 chimera.

**Discussion**

Taken together, our results demonstrate that the pore domain of the TRPV1 channel is a bona fide protein domain because it can be transplanted into the Shaker Kv channel without disrupting many functional properties of TRPV1 that have been attributed to the pore domain. Our C11 chimera retains activation by K2K2 and DkTx, has a higher permeability for small monovalent cations relative to NMDG+, and is permeable to Ca2+. This chimera is not robustly activated by external protons, even though protons are thought to bind within the outer pore (25, 47, 48). However, mutations outside the pore domain of TRPV1 can disrupt activation by protons (47), suggesting that transplantation of the pore from TRPV1 to Shaker might similarly perturb the allosteric network through which protons influence the closed-to-open equilibrium. Remarkably, the C11 chimera retains exquisite sensitivity to noxious heat, demonstrating that the pore domain of TRPV1 contains the elements required for producing steeply temperature-dependent activation. The steep increase in macroscopic current upon heating in TRPV1 arises from a steep increase in open probability rather than in the single channel conductance (8). Although we cannot distinguish between these two possibilities in our experiments with C11, we consider that the similarity in the temperature-activation relations between C11, TRPV1, and C23 makes it unlikely that their actions relative to NMDG+ and is permeable to Ca2+. This chimera is not robustly activated by external protons, even though protons are thought to bind within the outer pore (25, 47, 48). However, mutations outside the pore domain of TRPV1 can disrupt activation by protons (47), suggesting that transplantation of the pore from TRPV1 to Shaker might similarly perturb the allosteric network through which protons influence the closed-to-open equilibrium. Remarkably, the C11 chimera retains exquisite sensitivity to noxious heat, demonstrating that the pore domain of TRPV1 contains the elements required for producing steeply temperature-dependent activation. The steep increase in macroscopic current upon heating in TRPV1 arises from a steep increase in open probability rather than in the single channel conductance (8). Although we cannot distinguish between these two possibilities in our experiments with C11, we consider that the similarity in the temperature-activation relations between C11, TRPV1, and C23 makes it unlikely that their responses to heating arise from completely different mechanisms. Mutations throughout the sequence of TRPV and TRPA1 channels have been reported to alter activation of these channels by heat, including the pore domain (16, 17, 20, 49), the N-terminal Ankyrin-repeat domains (9, 10, 50–53), and the C terminus (12). However,
Fig. 4. Heat activation of TRPV1 and the C11 chimera. (A) Representative current traces for TRPV1-expressing cells activated by heating. Pulses from 0 to −60 mV were given at 2 Hz. (B) Current vs. temperature relations at −60 mV obtained from experiments as in A for temperatures from 20 °C to 50 °C, with the mean current values at the end of each pulse to −60 mV displayed as a line graph. Individual cells are shown with currents normalized by their amplitude at 36 °C (n = 24). (C) Plot of log (Itest/I36 °C + 1) obtained from the relations in A with the mean relation shown as red circles ± the SEM. The ratio of Itest/I36 °C was offset by arbitrarily adding a value of 1 to the data to avoid large distortions in the log curves at lower temperatures, resulting from having small leak currents where some data points are ≤0. The linear fit at low temperatures (dotted black line) had a Q10 of 1.1, while that at high temperatures (continuous black line) had a Q10 of 26.8. The inset shows Q10 values for low and high temperatures from fits to data from individual cells (gray circles) and red circles representing the mean values ± the SEM in black. (D–F) Representative current traces, temperature-activation relations, and Q10 determinations for cells not expressing exogenous channels (n = 88). (G–I) Representative current traces, temperature-activation relations, and Q10 determinations for cells expressing the C11 chimera (n = 108). The fit at low temperatures had a Q10 of 1.3, while that at high temperatures had a Q10 of 40.8. (J) Representative current time course for C11 obtained similarly as in G, showing Zn2+ block of temperature-activated currents. Temperature is shown in red on the Upper Inset, and it was increased from room temperature to ∼42 °C, after which a solution supplemented with 2 mM Zn2+ at an equally high temperature was applied (denoted by the thick black horizontal line). The dotted blue line denotes the zero-current level. Data from a population of cells was pooled after normalizing the initial current at room temperature (1) and the current after Zn2+ application at high temperature (3) to the current at an equivalent temperature before Zn2+ application (2). The resulting mean ± SEM for both temperature (x axis) and current (y axis) (n = 3) are shown on the Inset to the right, where the y axis denotes normalized current and the x axis the temperature in which the pooled data were obtained. (K) Representative current time courses showing C11 (continuous gray curve) activation by heating, followed by deactivation upon cooling, obtained similarly to G. Temperature for the C11 experiment is shown as a continuous red curve at the Top. The dotted gray and red curves represent the current and temperature time courses for an uninjected oocyte from the same batch of oocytes as the C11 recordings. The blue dotted line denotes the zero-current level. Population data for C11 was pooled by normalizing the current at low temperature following heat activation (3) and the current at an equivalent temperature before heating (1) to the peak current measured at the maximal temperature (2), and is presented in the Inset on the Right. Data are shown as mean ± SEM for both temperature (x axis) and current (y axis) (n = 3). (L–N) Representative current traces, temperature-activation relations, and Q10 determinations for cells expressing the C23 chimera (n = 16). The fit at low temperatures had a Q10 of 1.2, while that at high temperatures had a Q10 of 29.8.
Fig. 5. A mutation within the pore domain of TRPV1 similarly alters gating of TRPV1 and the C11 chimera. (A) Time course for activation of membrane currents following application of capsaicin for cells expressing TRPV1 F640L (Left) or K2K2 for cells expressing C11 F640L (Right). Cells were held at −60 mV, and voltage was stepped to +60 mV for 100 ms every 3 s. TRPV1 was subsequently inhibited by RR (50 μM; Left) and the C11 chimera was inhibited by Zn²⁺ (2 mM; Right). (B) Normalized I–V relations for control (black symbols), capsaicin (10 μM, red symbols, Left), K2K2 (red symbols, Right), Ruthenium Red (10 μM, blue symbols, Left) or Zn²⁺ (2 mM, blue symbols, Right) obtained from experiments as in A. Currents were normalized to the amplitude of current in the presence of 10 μM capsaicin or 5 μM K2K2 at +60 mV (mean ± SEM for n = 3–5). (C) Representative current traces obtained by stepping from 0 mV to −60 mV for heat activation of TRPV1 F640L (Left) and C11 F640L (Right). (D) Temperature-activation relations for cells expressing TRPV1 (gray; n = 7), TRPV1 F640L (black; n = 11), C11 (gray; n = 7), and C11 F640L (black; n = 13). Constitutive current measured at low temperature (15 °C for TRPV1 and 20 °C for C11) was subtracted and currents were then normalized to the amplitude of current at 47 °C (TRPV1) or 45 °C (C11).
For experiments in Fig. 5A, the C11 chimera and TRPV1, Ca2+ KCl, 5 mM Hepes, 1 mM MgCl2 and 1.8 mM CaCl2, 0.01 M DMSO, 1 mM ATP, 50 mM gentamicin, pH 7.6 with NaOH using the two-electrode voltage-clamp recording technique (OC-725C, Warner Instruments) with a 150–4-L recording chamber. Data were filtered at 1 kHz and digitized at 5–10 kHz using pClamp software (Molecular Devices). Microelectrode resistances were 0.1–1 MΩ when filled with 3 M KCl. For recording macroscopic membrane currents in most experiments, the external recording solution contained (in millimoles/liter): 100 KCl, 10 Hepes, pH 7.6 with KOH at room temperature (−2 °C). For experiments to assess the Ca2+ permeability of the C11 chimera and TRPV1, Ca2+ (1 or 3 mM) was added to the external solution. In some experiments, 40 mM BAPTA was injected to prevent activation of calcium-activated chloride currents. If we assume an oocyte volume of 500 nL, the final intracellular concentration of BAPTA would be ∼4 mM.

For temperature-activation experiments, calcium-activated chloride currents were minimized by using calcium-free solution containing 30 μM Cacinch-A01. External solutions were passed through glass capillaries spilters immersed in a water bath maintained at about 70 °C, and recordings were performed during spontaneous perfusion with temperature measured using a thermistor located close to the cell. Oocytes expressing TRPV1 and C11 were only studied if uninjected oocytes from the same batch contained a low density of endogenous temperature-sensitive currents at negative membrane voltages. For experiments testing for Zn2+ inhibition (Fig. 4J) or reversibility of temperature-activated currents (Fig. 4K) we used several perfusion lines to independently perfuse Zn2+ ions at high temperature or cold solution in which the perfusion capillaries were passed through a bath of ice water before reaching the recording chamber. O2 values were obtained from Q2=10mV, where m is the linear slope of log(VO2/1–V). In the temperature intervals were fixed and the fits were done automatically using a script for IgorPro 6.3A4A (Wavemetrics). There were no differences in using the automated vs. manual fitting for the control data, which was essentially temperature independent. The mean log(V02/1–V) was calculated by automatically binning each of the recordings in temperature intervals of 0.5 °C and averaging a single data point within each interval per cell.

ACKNOWLEDGMENTS. We thank Alex Cherler, Joe Mindell, Shai Silberberg, Gilman Toombes, and members of the K.J.S. laboratory for helpful discussions; and Edoardo Sarti for assistance with TM-align. This work was supported by the Intramural Research Programs of the National Institute of Neurological Disorders and Stroke (NINDS), NIH (to K.J.S.), by a NINDS Competitive Postdoctoral Fellowship and K99 Career Development Award (to A.J.-O.), and by a grant from the Korea Research Institute of Bioscience and Biotechnology Research Initiative Program (Korean Biomedical Scientist Fellowship Program) (to C.B.).
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